Altered electrophysiologic and pharmacologic response of smooth muscle cells on exposure to electrical fields generated by blood flow

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ABSTRACT The flow of the blood past the vascular wall gives rise to an electrical potential. This field is calculated to have a periodic waveform with a transluminal peak-to-peak amplitude of ~ 1.35 V/m^{-1}. Digital imaging fluorescent microscopy was used to measure changes in the membrane potentials of smooth muscle cells by following changes in the fluorescence of the potential sensitive dye, 3,3'-dipropyloxacarbocyanine iodide (di-O-C_5[3]). The effect of the low level electrical field on the membrane potentials of cultured smooth muscle vascular cells was shown to cause a steady-state depolarization of ~ 10 mV. The degree of steady-state depolarization was shown to directly vary with the frequency of the applied field and the effect was not dependent on the presence of extracellular Ca^{+2} or Mg^{+2}. These effects are thought to be most consistent with an electroconformational coupling mechanism. The presence of this electrokinetic field was also shown to alter the electrophysiological response of smooth muscle cells treated with 5-hydroxytryptamine. Cells exposed concurrently to both 5-HT and the electrical field showed an increased membrane depolarization thus implying that the electrokinetic field may be important in both normal and pathologic cellular responses.

INTRODUCTION

Smooth muscle cells, as key cellular elements in the arterial vasculature, are exposed to a wide variety of stimuli because of their proximity to the blood stream. Among these is an endogenous electrical field resulting from the movement of the blood past the blood vessel wall. This electrokinetically generated field is the streaming potential; its existence has been demonstrated previously in mammalian vasculature (1–3). Based on an estimate of this field strength by a simple mathematical model, the effect of these fields was studied in a cell culture model of aortic smooth muscle cells.

The magnitude of electrical field generated by blood flow was calculated based on the classical derivation of the streaming potential as described by the Helmholtz-Smoluchowski equation (4, 5), with a correction for pulsatile flow, $f(Y_a)$, as described by Packard (6):

$$E_S = [\zeta\epsilon\epsilon_0 P/\eta\kappa]\,[f(Y_a)],$$

where E_S is the streaming potential (volts), ζ is the zeta potential (volts) of the vessel, ϵ is the dielectric constant of the electrolyte, ϵ_0 is the permittivity of free space, P is the effective systolic pressure in N/m^{-2}, η is the electrolyte viscosity in kg/m^{-1}/s^{-1}, and κ is conductivity in Siemens/m^{-1}. The waveform was treated as sinusoidal. The Helmholtz-Smoluchowski treatment makes three assumptions, (*a*) it ignores the possibility of surface conduction, (*b*) it mathematically neglects the thermally derived diffuse arrangement of ions in solution, and (*c*) it assumes unvarying and laminar flow. In this model, neither significant surface conduction nor the diffuse arrangement of the ions in the electrolyte phase were taken into consideration, although these modifications could be incorporated after the work of Rutgers and Neale, respectively (7, 8). Laminar flow was assumed after MacDonald (9) although this assumption will fail when the vessel wall is disrupted, for example, by injury or deposits of atherosclerotic plaque.

The magnitude of the streaming potential in an animal model of the neonatal rat aorta was calculated using a ζ potential value of -200 mV after Sawyer (3), a viscosity of 3.5×10^{-3} kg/m^{-1}/s^{-1}, a dielectric constant of 20 (10), an effective pressure of 9.403×10^3 N/m^{-2} at a rate of 2 H, with the conductivity of the blood given a value of 0.67 S/m^{-1}. Neonatal rat aortas measure ~ 0.5 mm in diameter, thus the correction factor given by $f(Y_a)$ is 1.0. This gives a magnitude for the effective streaming potential of 1.89×10^{-4} V (the peak to peak potential is 2.67×10^{-4} V). This calculation agrees in magnitude with previously measured streaming potentials (1, 2). The transluminal distance in the neonatal rat aorta is ~ 0.2 mm and consequently the smooth muscle cells resident in the walls of the vessel will be subject to a transluminal electrical field strength of 1.34 V/m^{-1} p-p or 0.945 V/m^{-1} RMS.

It was of interest to study whether the electrophysiology and electropharmacology of vascular smooth muscle cells would be affected by such fields. The possibility that changes in the membrane potential might be induced was especially intriguing because of the association

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between hypertension and derangements of the membrane potential and membrane ion transport (11–13). Therefore, a method using the membrane potential sensitive fluorometric dye, 3,3′-dipropyloxacarbocyanine iodide (di-O-C_5[3]) to measure changes in membrane potential (14–16) was adapted for digital imaging fluorescence microscopy of a system of cultured aortic smooth muscle cells. It is shown here that the exposure of these cells to the electrokinetically modeled low magnitude alternating electrical fields leads to a depolarization of the DC membrane potential and also alters the normal electrophysiologic response to the vasoactive agent, 5-hydroxytryptamine.

METHODS

Smooth muscle cell cultures

Neonatal rat smooth muscle cells were isolated from aortae of 1–3 d old Sprague-Dawley rats as described previously (17–18). Briefly, a collagenase-elastase solution in 20 ml of Dulbecco's modified Eagle's media (DV) was added to the medial layer of 20 aortae that were finely minced after the careful dissection. After digestion at 37°C for 45 min, the resulting cell suspension was centrifuged and the pellet was washed twice with DV containing 3.7 g/l sodium bicarbonate, 20% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. After washing the cell pellet was resuspended in fresh medium and seeded at a density of 5.0×10^5 cells/25 cm^2 cell culture flask (Dow Corning, Corning, NY). Cells in primary culture were maintained for 7 d at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were subcultivated after trypsinization at a reseeding density of 2.0×10^4 cells/cm^2 into observation chambers. Imaging chambers were made by drilling 0.8 cm holes cell culture trays (Dow Corning #25820), and then, using an epoxy glue, a #1 microscope cover slide was attached to the bottom of the tray. The epoxy was cured at 37°C for 24 h, and then the glass window was acid washed with 0.1 N HCl for 30′ at 25°C followed by exhaustive washing with purified water. The chambers were sterilized by exposure to a germicidal UV lamp for 2 h and then were covered and stored for use within 72 h in cell culture experiments.

Imaging system

Microfluoroscopic analysis of the cells was conducted with an inverted Nikon Diaphot microscope equipped with epifluorescent optics. The fluorescent images were captured through a video-camera port by a Dage-MTI 66 video camera with a grade II silicon intensified vidicon tube. The images were passed in parallel into a Panasonic model 8050 time-lapse video recorder, used for data backup and continuous archiving, and also into an Imaging Technologies, Inc. (Woburn, MA). PCVision Plus frame grabber board that was resident in an IBM XT computer. Image processing was performed by using ImageLab software from Werner Frei Associates (Santa Monica, CA) and image analysis was done using ImageMeasure software from Microscience, Inc. (Federal Way, WA). Certain image integration and differential analyses not available in the above software packages were custom written in the laboratory. All of the hardware and software ran on an IBM XT microcomputer configured with 640 K RAM, 8087 math coprocessor, EGA graphics board and monitor and 40 MByte hard disk. A Sony Trinitron analogue RGB monitor (model #PVM-1271Q) driven directly by the ITI imaging board was used as the image monitor. For fluorescent imaging experiments, the intensity, accelerating potential, and black level controls of the SIT camera were adjusted to ensure operation of the camera in the linear response range. Appropriate neutral density filters were placed in the light path of the mercury vapor arc light source to attenuate the excitation intensity of the light during experiments and to ensure that measurements remained in the linear range of the system. Cells were only illuminated during image acquisition and the light source was shuttered at all other times. Image processing was done as postprocessing. Images were background subtracted with the resultant image then either used directly for analysis or after reduction of background noise with a binary mask.

Electrical stimulation

The sinusoidal electrical waveform was generated by a signal generator (Beckman Industrial Corp. Circuitmate-model #FG2, Brea, CA) whose output signal was fed through a unity gain follower circuit. The output signal, routinely monitored by an oscilloscope and a digital multimeter. This signal was fed into a pair of platinum electrodes separated from the cell culture system by a pair of salt bridges made either of 0.3 M NaCl in agar or of hydroxyethylmethacrylate (19). The actual resistance across the cell culture chamber was 250 Ω and the application of a sinusoidal 2 Hz current of 40 μA peak to peak results in a stimulation field strength of 1.25 V/m^{-1} peak-to-peak. Because the power dissipated in these experiments calculates to 2.8 μW, it was not considered necessary to monitor the temperature of the cell chamber for Joule heating.

Fluorometric determination of membrane potential

Membrane potentials of the smooth muscle cells were monitored by measuring changes in the fluorescence of the cyanine dye 3,3-dipentyloxacarbocyanine [di-O-C_5(3)] (Molecular Probes, Inc. Eugene, OR). Aortic smooth muscle cells were washed with Puck's saline G (137 mM NaCl, 5 mM KCl, 1.1 mM KH_2PO_4, 2 mM Na_2HPO_4, 6 mM glucose, pH 7.4) and then placed in a final volume of 1 ml Puck's Saline G supplemented with 1.1 mM $CaCl_2$ and $MgCl_2$. The cells were loaded with Di-O-C_5(3) at a final concentration of 1×10^{-8} M for 20 min at 37°C. Changes in the fluorescence of cells (ΔF) were determined in di-O loaded cells by measuring the integrated intensity of each cell both initially (F_o) and after stimulation (F). The normalized change in fluorescence ($\Delta F/F_o$) for each cell was then calculated and expressed as a percent change in fluorescence. All experiments were performed with the intermittent shuttering of the excitation light because control experiments showed that photobleaching of the dye did not occur even with multiple intermittent exposures over the time course of the experiments.

Resting membrane potentials of the smooth muscle cells were determined by adding the ionophore valinomycin to cells bathed in varying extracellular concentrations of K^+ (14). Di-O loaded cells were bathed in Puck's containing both Ca^{+2} and Mg^{+2} and various extracellular potassium concentrations used of 6, 25, 50, 75, and 100 mM K^+ with each solution made isotonic by the appropriate addition of NaCl. Fluorescent images of cells were obtained using an excitation wavelength of 485 nm and observing the emission at 520 nm. After the acquisition of an initial fluorescent image, (F_o), the smooth muscle cells were made permeable to potassium by the addition of valinomycin (Sigma Chemical Co., St. Louis, MO) at a final concentration of 7.5×10^{-6} M. In some initial experiments, images were obtained every 15 s after the addition of valinomycin to determine the relaxation time required to reach a new steady state potential. This relaxation time

was found to occur within ~90 s, and consequently, in all subsequent experiments, the final fluorescent images were obtained at 2–3 min.

The electrophysiological response of the cells to 5-hydroxytryptamine stimulation was measured at varying doses, and the membrane potential changes were followed by the normalized change in fluorescence. After initial fluorescence images were obtained, 5-hydroxytryptamine was added to each cell culture at doses that varied from 1×10^{-7} M to 1×10^{-3} M final concentration. Multiple images were taken over time until the fluorescent signal reached a new steady state, usually within 2 min.

Changes in the membrane potential of cells under the conditions of electrical stimulation were determined in di-O loaded cells by measuring the integrated intensity of each cell both before (F_o) and during electrical stimulation. The normalized change in fluorescence ($\Delta F/F_o$) for each cell was then calculated and expressed as a percent change in fluorescence. The response of cells to 5-hydroxytryptamine during the stimultaneous exposure of the cells to the low level electrical field was measured by obtaining a preelectrical-stimulation fluorescent image, then activating the electrical field and establishing the new baseline membrane potential. With exposure to the electrical field being maintained, the response curve of the membrane potential to 5-hydroxytryptamine dose as described above was generated.

RESULTS

The use of neonatal rat smooth muscle cell cultures has been well established as a long term system for the production of insoluble extracellular matrix components, especially with respect to elastin and collagen (18). In these systems, adherence to the culture surface (Corning cell culture plastic) occurs within 4–6 h and spreading begins within 8 h. Division of the cells begins at ~20 h. Cells seeded at the same density as those in these experiments become confluent within ~96 h. The cells respond in an identical fashion on the glass surface of the modified imaging/culture system at least up to 96 h. The experiments using fluorescent microscopy examined cells that were spread but dispersed, without cell–cell contact. Consequently, the experiments were always performed within 24–48 h of seeding.

The fluorescence of the cationic cyanine dye, di-O-C_5-[3] depends on the presence of a hydrophobic/lipophilic environment, and hence fluorescence of the molecule is observed when the dye partitions into the membrane. The dye concentration, and hence the intensity of the fluorescence, depends on the transmembrane potential. The resting membrane potential of smooth muscle cells though not determined by a simple Nernstian relationship between the internal and external potassium concentration (20), is dependent on the potassium concentrations as Fig. 1 demonstrates. With the addition of valinomycin, the K^+ equilibrium potential was expected to dominate though at physiological electrolyte concentrations other workers have shown that the membrane potential deviates from a potassium potential dependence and thus would be expected to exhibit a more complex behavior (20, 21). The experimental data were consistent with this analysis (Fig. 2). Using the potassium dominated portion of the experimental curve and extrapolating a line of slope ($\Delta F/F_o/\log[K^+]$) to zero gives a resting membrane potential of ~41 mV for the aortic smooth muscle cells which is in reasonable agreement with the values reported by other methodologies (22). A 1% change in the normalized fluorescence corresponded to approximately to a 1-mV change in membrane potential, which is consistent with the reported literature values for di-O (14–16).

When cultured smooth muscle cells were exposed to a model electrokinetic field (a 2 Hz sinusoidal field of 1.25 V/m^{-1} peak-to-peak), a steady-state DC depolarization of the cells was consistently found. In eight separate experiments involving the imaging and analysis of 68 cells, the steady-state fluorescence change was $-9.11 \pm 3.89\%$ corresponding to a depolarization of ~10 mV. This DC depolarization did not depend on the presence of extracellular Ca^{+2} or Mg^{+2} because removal of these cations from the extracellular medium did not alter the observed depolarization. When the magnitude of the applied field was varied, a minimum threshold for the depolarization effect could be demonstrated. No depolarization of the membrane was found when the field strength was <0.75 V/m^{-1}, although a significant graded response in terms of increasing depolarization at field strengths up to 6.7 V/m^{-1} was not seen. Experiments in which the frequency of the applied field were varied showed a direct relationship between the frequency of the applied field and the degree of steady state depolarization (Fig. 3).

The vasoconstriction of a blood vessel induced by 5-hydroxytryptamine (5-HT) is a well-described response to vessel injury that involves membrane depolarizations and contraction of the muscle fiber (23–25). As Fig. 4 *a* shows, when the di-O loaded cells were treated with 5-HT at physiological doses known to cause membrane depolarizations, a dose-dependent depolarization of the cells could be demonstrated. This baseline response of the cells to 5-HT was altered when smooth muscle cells were concurrently exposed to the modeled-electrokinetic field. As Fig. 4 *b* shows, the presence of the field significantly potentiated the magnitude of the measured depolarization and altered the shape of the dose-response curve.

DISCUSSION

A role for electrical field interaction with cells leading to effects on the development and functioning of a wide variety of biological systems have been described (26).

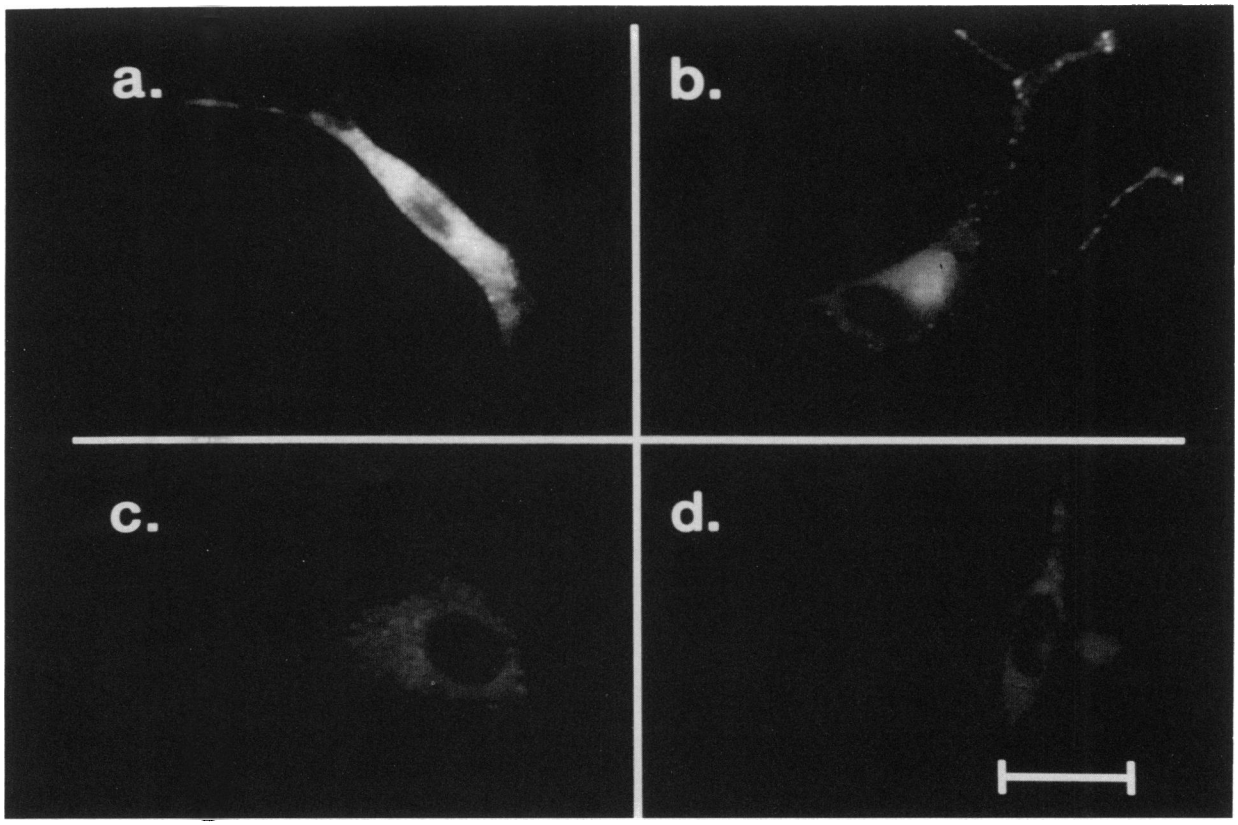

FIGURE 1 Representative di-O loaded neonatal rat smooth muscle cells in extracellular buffer media as described in the text. The potassium concentrations are as follows: A = 6 meq; B = 26.5 meq; C = 53 meq; and D = 79.5 meq. Cells have been magnified 800X, images shown have been enhanced by arithmetically averaging five video frames and then capturing these images on a video film recorder. The bar represents 10 μ.

There are a number of mechanisms that have been proposed to account for these bioelectric effects including the direct induction of membrane potential changes caused by coupling of the membrane to an external electric field (27, 28), in situ electrophoresis of membrane components (29, 30), and alteration in the functioning of membrane bound ion channels and enzymes due to variations in the ion activities induced by the external electric field (31–35).

After the analysis of Cole for a prototypic spherical cell (28), the calculated transmembrane potential alteration directly induced by the electrokinetic field is ~50 μV which is inconsistent with the measured fluorescence change thus making this mechanism unlikely as an explanation for the effects reported here. While reorganization of membrane components by in situ electrophoresis has been established at field strengths as low as 0.02 V/m^{-1} (36), such effects require a significant duration of exposure (hours) to the electrical field thus also making this an unlikely mechanism for the observed depolarization. Furthermore, with a sinusoidal field, a net electrophoretic motion of membrane components would be unexpected.

The coupling of applied periodic electric fields to membrane-associated enzymatic processes has been established both theoretically and experimentally (31–35). This mechanism, electroconformation coupling, is applied to the conformational transitions in a macromolecule that involve portions of the molecule having intramolecular separations of charge or altered dipole moments that respond to changes in the electric field across the membrane. The principle of electroconformational coupling is that a chemical reaction, even when catalyzed by an enzyme, may proceed at a relatively negligible rate until the field-induced conformational change of the enzyme activates the enzyme and substantially increases the k_{cat}. Enzymatic electroconformational coupling is generally predicted to be frequency specific, and experimental evidence supports these predictions. Importantly, while studies of the altered activity of the membrane associated Na^+, K^+-ATPase in AC fields have been shown to have a frequency dependence (34, 35), a

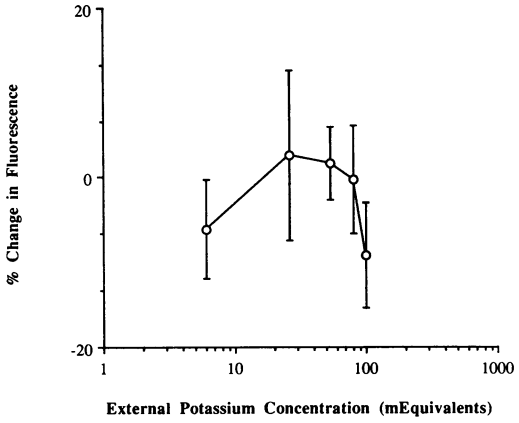

FIGURE 2 Change in fluorescence for smooth muscle cells in different extracellular media after treatment with valinomycin. Experimental data from a representative valinomycin experiment as described in the text. Each data point plotted represents the mean of the fluorescence change for five sets of individual cells each containing eight cells. Error bars show the standard deviation of the data. It is assumed that a 1% change in fluorescence is equivalent to a 1-mV change in membrane potential.

voltage dependence of the effect is not always clearly demonstrated above a certain threshold (34). Given the frequency and voltage data observed in the smooth muscle cell experiments, invoking electroconformational coupling as the mechanism provides a reasonable explanation for the experimental observations.

In addition to altering the electrophysiologic state of cells, the presence of the electrokinetic field altered the response of smooth muscle cells to other native stimuli. It is presently unclear if the 5-HT potentiation is secondary to the altered membrane potential or is an independent effect of an electroconformational coupling process acting at the serotonin receptor. Altered membrane potentials, secondary to a loss of electrogenic Na^+ and K^+ pumping, have been shown to increase sensitivity of vascular smooth muscle to vasoactive agents (12). These experiments show more than an increased sensitivity to 5-HT because the maximum depolarization seen at supra-maximal doses of 5-HT is greatly exceeded in the presence of the electrical field. An electroconformational coupling mechanism, acting at the level of the serotonin receptor to alter either binding or rate constants is a possible candidate for these effects.

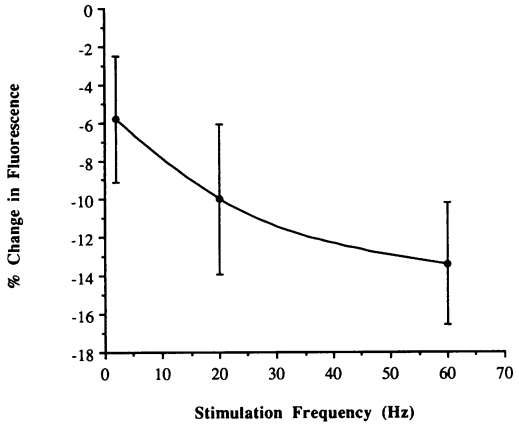

FIGURE 3 Changes in membrane potential as a function of the frequency. The effect on smooth muscle cell membrane potential as determined by measuring $\Delta F/F_o$ with varying the frequency of a field of 1.25 V/m^{-1}. The curve shown is a representative experiment of eight individual cells. Each data point represents the mean of the change in fluorescence and error bars show the standard deviation for each point. The differences between the means represented by each point are significant to better than $p < 0.05$.

The response of the vascular smooth muscle cells even in this simplified model provides evidence that the presence of electrokinetic low potential fields are of possible importance both in the normal functioning and the pathologies of the vascular system. Among the obvious refinements of the model is the incorporation of an electrical waveform that results from a pressure wave that more accurately represents the actual pattern of aortic blood flow. Interestingly, although the actual waveform of the aortic pressure pulse is a systolic triangular wave with an amplitude that is positive except for a short negative portion that occurs during protosystole, and a flow of zero during diastole, such a refinement may not be so significant because in experiments done by Frindl and Kurtz (37) with a left ventricular/aortic stimulator, the transluminal pulses generated in a model aorta by a triangular pulse of fluid resulted in a biphasic triangular wave that has significant sinusoidal character. Another important consideration is the effect of these stimuli with chronic exposure. Since cells in the in vivo environment must always be exposed to such stimulation, questions about the modification of development and normal function are important. In an environment of chronic exposure, it is likely that effects such as the in situ electrophoresis of membrane components may play a more substantial role. Smooth muscle cells play a dual role in the vasculature providing both vascular tone and generating extracellular matrix through their contractile and synthetic functions, and it is possible that the presence of the persistent streaming potential fields may modify not only their response to vasoactive events but also to the production and accumulation of extracellular matrix.

It is tempting to speculate briefly on potential clinicopathological correlations to in vivo exposure to these low level fields. The electrokinetic model is sensitive to changes in the parameters of pressure, laminar flow, and

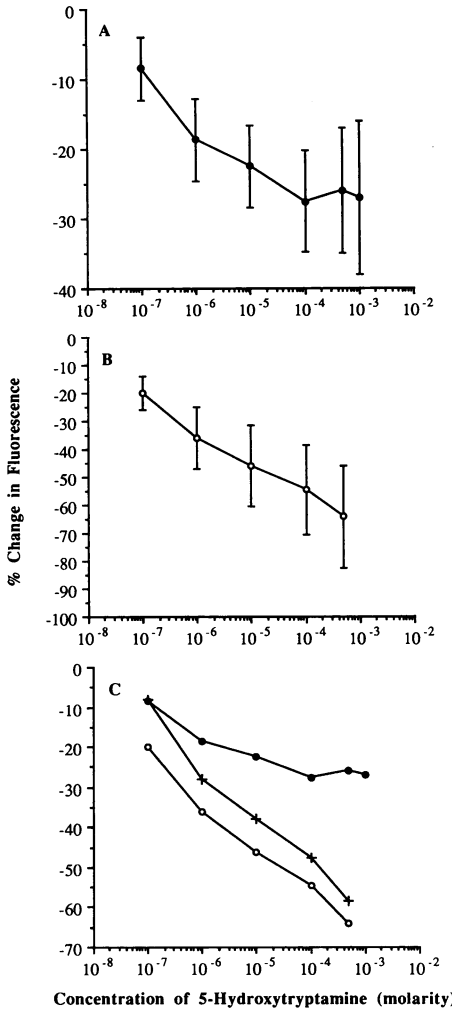

FIGURE 4 The graph in *a* shows the effect of 5-hydroxytryptamine on the membrane potential of the neonatal rat smooth muscle cells as indicated by measuring the $\Delta F/F_o$. *a* shows that supramaximal doses of 5-HT led to depolarizations on the order of 30 mV which is in agreement with the results of other laboratories as described in the text. The graph in *b* shows the potentiation of the 5-HT depolarization effect in cells that are concurrently being stimulated with a 1.25 V/m^{-1}, 2 Hz sinusoidal electrical field. Each curve shown is a representative experiment of eight individual cells. Final concentrations of 5-HT are shown. Each data point represents the mean of the change in fluorescence and error bars show the standard deviation for each point. In *c* two curves representing the raw data (●, ○) in which the changes in fluorescence are normalized to the unstimulated cell ($\Delta F/F_0$), and a difference curve (+) in which the changes in fluorescence are normalized to the electrically stimulated cell ($\Delta F/V_0$) are compared with the 5-HT dose response of a set of unstimulated cells. For the raw data, the differences between the means for each case (unstimulated vs. stimulated) are statistically significant for $p < 0.0003$. The differences between the means for the difference data ($\Delta F/V_0$) between the two cases are not significant for 5-HT concentrations of 10^{-7} M and 10^{-6} M but are significant at better than $p < 0.02$ for the remaining concentrations.

zeta potential. Raising the hydrostatic pressure, which occurs in hypertension, can lead to a change in the intensity of the electrical field imposed across the vessel. Because a threshold field strength is required to elicit the depolarization effect, it is possible that under normotensive conditions, the field strength is below threshold and only in hypertension are field strengths reached capable of effecting changes such as those seen with the serotonin potentiation. Furthermore, the long term effects of the electric fields at various magnitudes are unknown at present. Turbid flow, such as would be caused in the blood stream by an atherosclerotic plaque, will substantially increase the magnitude of the streaming potential (37). Alterations in the ζ potential could result from injury to the negatively charged endothelium even to the extent that sign reversal could occur if the endothelium were denuded, thereby exposing the underlying extracellular matrix made predominantly of elastin and collagen, both proteins with a strong net positive charge. If the smooth muscle cells were to respond to these altered fields by increasing their proliferation or their accumulation of extracellular matrix, events well recognized to occur in atherosclerosis, this might indicate a role for the streaming potentials in histopathological events. Perhaps most intriguing, a contributing role to the vasospasm associated with coronary arterial disease is suggested since as the serotonin experiments show, the presence of the appropriate electrical field will alter the smooth muscle cell's response to vasoactive agents. Because vasospasm often occurs near areas of atherosclerotic plaques, the local alterations in streaming potential field caused by the altered flow might make those smooth muscle cells either react paradoxically or hyperreact to vasoactive agents causing paradoxical or increased vasoconstriction. Such a mechanism might conceivably contribute to the observations concerning paradoxical vasoconstriction in coronary artery atherosclerotic lesions (38–40).

In conclusion, although the model and experimental mechanism for the interaction of streaming potential generated electrical fields with smooth muscle cells is at an elementary stage, understanding this phenomena may have important implications both in the knowledge of how electrical forces influence bioelectrochemical events and in the understanding of clinically relevant pathophysiology.

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