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Activation of human neutrophils by electronically transmitted phorbol–myristate acetate

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Summary We report the transfer of the activity of 4-phorbol-12-β-myristate-13-acetate (PMA) by electronic means. Neutrophils were placed at 37°C on one coil attached to an oscillator, while PMA was placed on another coil at room temperature. The oscillator was then turned on for 15 min, after which cells were usually further incubated for up to 45 min at 37°C before measurement of reactive oxygen metabolites (ROMs) production. In 20 blind experiments, PMA thus 'transmitted' induced ROM production. ROM were not induced when: (1) PMA vehicle or 4-α-phorbol 12,13-didecanoate (an inactive PMA analogue) were transmitted; (2) the oscillator was switched off; (3) superoxide dismutase or protein kinase C inhibitors were added to cells before transmission. These results suggest that PMA molecules emit signals that can be transferred to neutrophils by artificial physical means in a manner that seems specific to the source molecules. © 2000 Harcourt Publishers Ltd

INTRODUCTION

Neutrophils participate in host defense against bacterial, fungal and viral infections by phagocytosing and killing invading bodies. The killing depends largely on activation of the respiratory burst provoking the formation of reactive oxygen metabolites (ROMs) consisting of superoxide anions, hydroxyl radicals, hydrogen peroxide and singlet oxygen (1). The respiratory burst can be initiated by a variety of stimuli including tumor necrosis factor α, the chemotactic peptide fMLP and 4-phorbol-12-β-myristate-13-acetate (PMA) (2–4). Most of these interact with target cell receptors which activate a cascade of biochemical reactions. PMA, which can mimic, in vitro, a number of activation events occurring during inflammation, owes its effect to the fact that it is a potent activator of protein kinase C (PKC), a key element in a neutrophil activation cascade.

These chemical reactions within and between cells are reactions between molecules and thus ultimately driven by physical forces. Moreover, it is not insignificant that a growing number of observations suggest the susceptibility of biological systems to electric, magnetic and electromagnetic fields, with electric fields able to drive enzymes to do work, and cellular functions, including DNA and RNA synthesis, being modulated by low-frequency electromagnetic fields (5–9). These considerations informed exploratory research which led us to speculate that biological signaling might involve low-frequency waves potentially transmissible to cells by artificial, physical means. In the present study, we attempted the electronic transfer of PMA activity to neutrophils, which were thereby stimulated to produce ROM as though the PMA were physically present.

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MATERIALS AND METHODS

Reagents

Plasmagel was obtained from Laboratoire R. Bellon, Neuilly/Seine, France; ultra-pure water, HEPES solution (1 M), phenol red-free Hank's balanced salt solution (HBSS) without Ca2+ and Mg2+, pH 7.4 from Biochrom, Berlin, Germany; Ficoll-Paque from Pharmacia Fine Chemicals, Uppsala, Sweden; fatty acid- and endotoxin-

free bovine serum albumin (BSA) from Miles, Kankakee, IL, USA; cytochrome c (horse heart, type III), lipopolysaccharide (LPS) from *Escherichia coli* strain O111:B4, DMSO (dimethyl-sulfoxide), superoxide dismutase (bovine E, SOD), PMA (4-phorbol-12-β-myristate-13-acetate), 4-α-PDD (4-α-phorbol 12,13-didecanoate) from Sigma Chemicals, St Louis, MO, USA. PMA or 4α-PDD were dissolved in DMSO at 10 mM and stored at −20°C. Vehicle (DMSO from the same batch) was also aliquoted and stored at −20°C. Immediately before use, the stock solutions were diluted to appropriate working concentrations in ultra-pure water. PMA and 4α-PDD were used at 1μM while their 'vehicle' consisted of DMSO at the same concentration (0.06%, v/v) as that present in the respective solutions. The PKC inhibitors H-7 was obtained from Biomol Research Laboratories, Plymouth, PA, USA, and GF109203X (10) was kindly provided by Dr F. Russo Marie (INSERM U332, ICGM, Paris, France). All plastic materials were sterile and purchased from Becton-Dickinson, Oxnard, CA, USA.

Preparation of human neutrophils

Human blood from consenting healthy donors was anticoagulated with citric acid–dextrose (ACD). Blood was sedimented for 30–45 min in gelatin (Plasmagel) 0.3% (v/v) final. The supernatant was layered on Ficoll-Hypaque (density 1.077) and centrifuged (400 × g, 20 min, 20°C). The cell pellet was suspended in 1 ml of washing buffer (HBSS supplemented with 0.25% (v/v) BSA, 1 ng/ml LPS and 20 mM HEPES). Erythrocytes were lysed by adding 3 vol distilled water to the cell suspension, followed 40 s later by 1 vol of NaC1 3.5% (w/v). Cells were then washed twice, suspended in washing buffer and counted. All preparations contained at least 98% neutrophils as determined by microscopic observation after staining with May Grünwald–Giemsa (11,12). Cell viability as assessed by trypan blue exclusion always exceeded 95%. Neutrophils were suspended at 3×10^6/ml in washing buffer and kept at 37°C in a humidified CO_2 incubator until used (within 1–2 h). Before transmission or addition of ponderal agonists, neutrophils were suspended at 1×10^6/ml in washing buffer and Ca^{2+} (1.3 mM), Mg^{2+} (1 mM) and cytochrome c (80 μM) were added to the cell suspension which was then aliquoted (1 ml) into 1.5 ml Eppendorf tubes.

Transmission apparatus

The device used for transmission comprised a standard audio amplifier (Kemo Kit 105, West Germany) with magnetic coils connected respectively to the input an output (impedance 8 ohms). Tubes whose contents were to be transmitted were placed on the input coil and cells on the output coil. When the amplifier was not connected to the output coil, its output, as viewed with an oscilloscope, appeared to be noise with some 50 Hz contamination from the French power grid. However, when the amplifier was connected to the output coil, it behaved as an audio-frequency oscillator and signal analysis revealed the emission of a stable square wave with a frequency of about 3 kHz and voltage of approximately 7 V. In the presence of a weak, mV range signal not only the amplitude but also the frequency of the wave were modulated.

PMA transmission to neutrophils

For transmission experiments to cells, the input coil coupled to the amplifier was operated at room temperature, while the output coil was placed 2–3 m away in a 37°C humidified incubator. The source tube containing 10 ml of PMA (1 μM) or vehicle was placed on the input coil, and target tubes (capped, 1.5 ml Eppendorf tubes, usually in duplicate, containing neutrophils) on the output coil. The oscillator was then turned on for the 15-min transmission period. During the experimental procedure, the various parameters such as power, voltage, capacitance and impedance remained constant, the nature of the source tube (PMA versus vehicle) being the only variable.

For most experiments, four oscillators were used simultaneously. In all experiments, as additional control, tubes containing the same cell suspension as that placed on the output coil were placed 20 cm away from the output coil and referred to as 'unexposed cells'. After transmission, the oscillators were switched off and all cells were left in the incubator for the additional 0–45 min post-transmission incubation period, before OD measurement. In some experiments, superoxide dismutase (SOD, 40 μg/ml) or the PKC inhibitors, GF109203X (8 μM) or H-7 (20 μM) were added to cells 3 min before PMA or vehicle transmission. The positive control consisted of neutrophils directly stimulated by ponderal PMA (1 pM to 1 μM). Viability of all samples was assessed by trypan blue exclusion both before and after incubation.

Measurement of reactive oxygen metabolites

Following incubation, tubes were immediately placed on ice, pelleted in a microcentrifuge at 4°C (600 × g for 5 min) and supernatants transferred to cuvettes (Karlette Plastics, Adelaide, Australia). ROM production was measured as the reduction of cytochrome c using a spectrophotometer at 550 nm (Kontron, USA), as previously described (13). Results of transmission experiments are expressed directly as either absorbance (OD_{550}) or as percentage transmission defined as: 100 × (OD_{550} exposed cells–OD_{550} unexposed cells) / OD_{550} unexposed cells.

Table 1 Statistical analysis, summary

Experimental groups	Transmission (%)*	*P* value †	N° transmissions where T-PMA are all 'above' ‡	*P* value
T-PMA [T-vehicle]	47 ± 6 [8 ± 2.5]	$< 10^{-3}$	19 out of 20	2×10^{-5}
T-PMA [T-vehicle]	28 ± 5 [0 ± 2]	$< 10^{-3}$	20 out of 20	10^{-6}
T-PMA [T-PDD]	29 ± 13 [−2 ± 3]	2×10^{-2}	6 out of 6	2×10^{-3}
T-PMA on [T-PMA off]	24 ± 7 [−2 ± 1.4]	10^{-3}	11 out of 12	3×10^{-3}
All series	33.6 ± 3.4/2.3 ± 1.3	$< 10^{-8}$	56 out of 58	10^{-14}

ROM production as assessed at 60 min. Line 1: experiments 1 to 10 (Fig. 1); line 2: experiments 11–20 (Fig. 1). Raw data for T-PDD are shown in Table 2.

* Percent transmission was computed for each set of cells (cells exposed to either T-PMA, T-vehicle, T-PDD or T-PMA off). Data are presented as mean ± SEM.

† Differences between T-PMA cells and other experimental groups (control exposed cells) were analyzed using Student's *t*-test.

‡ The same data were also analyzed in a binary fashion. Each PMA transmission was thus classified as 'above' or 'below' the corresponding transmission of either T-vehicle, T-PDD, or T-PMA off. For all series except the third, two independent binary comparisons between means OD were performed. For the third series, one PMA mean was compared to two independent means (vehicle and PDD), so that the chance of obtaining a 'high' PMA was 1/3.

Statistical analysis

The statistical significance of the experiments was analyzed in two ways (Table 1). (1) Percent transmission (as defined above) was computed for each set of cells (cells exposed to either T-PMA, T-vehicle, T-PDD or T-PMA off). Data are presented as mean ±SEM. Differences between T-PMA cells and other experimental groups (control exposed cells) were analyzed using Student's *t*-test; (2) The same data were also analyzed in a binary fashion. Each PMA transmission was thus classified as 'above' or 'below' the corresponding transmission of either T-vehicle, T-PDD, or T-PMA off. Although this approach underestimates the statistical weight of each experiment, it has two advantages: (a) it is insensitive to the shape of the OD distributions; (b) it permits a pooling of experiments without loss of sensitivity, despite large fluctuations in absolute or relative OD values.

RESULTS

Electronic transmission of the PMA activity was carried out using a simple amplifier configured to function as an oscillator when coupled to two electromagnetic coils. On one coil was placed a source tube containing the PMA or its solvent ('vehicle'), while, on the other, in an incubator, were placed tubes containing the target neutrophils. In all experiments, several such oscillators were used simultaneously. A 15-min transmission period was followed by various post-transmission incubation periods with the oscillators switched off. ROM production was then measured as cytochrome c reduction.

Two series of ten consecutive, blind experiments were performed in which neutrophils were thus exposed for 15 min either to transmitted PMA (T-PMA) or to vehicle (T-vehicle) and OD_{550} measured 45 min later after a total incubation period of 60 min. For each experiment, four simultaneous transmissions were performed involving, respectively, two PMA and two vehicle source tubes. One of the two series of experiments was performed in a different laboratory (INSERM U332), with randomization and coding of source tubes being performed by the head of the laboratory. As shown in Figure 1, exposing cells to T-PMA resulted in an OD increase of 37 ± 4 % (mean ± S.E.M, $n = 40$ transmissions) compared to unexposed cells. By contrast, exposing cells to T-vehicle resulted in a 4.1 ± 1.8 % change. In the absence of cells, transmission of PMA or vehicle alone was without effect on cytochrome c reduction. Additional experiments showed that the PMA transmission effect occurred at a wide range of PMA concentrations in the source tubes. Exposing cells to T-PMA (1 nM in the source tube) resulted in an OD increase of 27 ± 2.9 % ($n = 4$ transmissions) compared to T-PMA (10 μM): 31 ± 14 %, not statistically different. Furthermore, the PMA transmission effect (24 ± 7 %, $n = 12$ transmissions) was absent when the oscillator was switched off (−2 ± 1.4 %). The contribution of superoxide to cytochrome c reduction was next examined. As illustrated in Figure 2, OD values at 60 min with and without SOD indicate that, upon PMA transmission, cytochrome c reduction was predominantly due to superoxide anions generated by the neutrophils.

The impact of T-PMA was also compared with that of an inactive analogue, 4-α-PDD, which was transmitted (T-PDD) for 15 min while OD values were measured after various post-transmission incubation periods. As shown in Table 2, an increase in OD was evident immediately after PMA transmission and, in most cases, remained apparent up to 60 min, although the magnitude and time course varied from blood-sample to blood-sample. By contrast, T-vehicle or T-PDD did not stimulate ROM production at any time point. The observation that

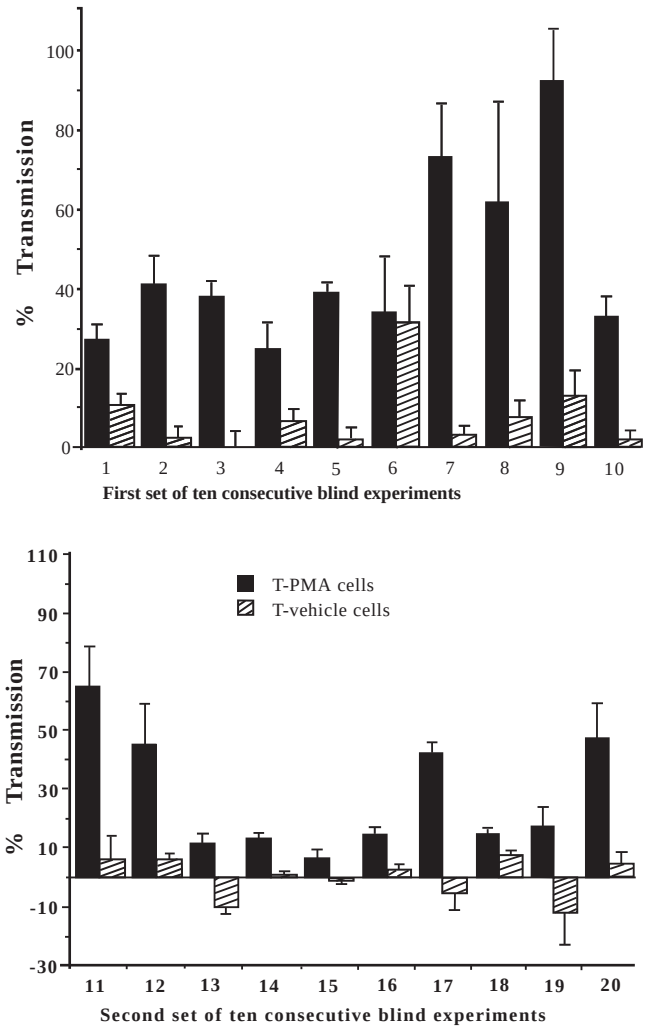

Fig. 1 Effect of transmitted PMA on neutrophil ROM production. Cells (1×10^6) were exposed at 37°C to either 'transmitted' PMA or 'transmitted' vehicle for 15 min. As an additional control, cells were placed 20 cm away from the output coil ('unexposed cells'). Incubation was continued for 45 min at 36°C before assessment of ROM production, as described in Materials and Methods. A first set of ten consecutive experiments (1–10) was performed at INSERM U200. A second set of ten consecutive experiments (11–20) was performed in a different laboratory (INSERM U332, ICGM, Paris). In each experiment, 4 simultaneous transmissions were performed, using 4 source tubes (2 PMA and 2 vehicles). These 4 source tubes were prepared, randomized and blinded by coding at the beginning of each experiment. In nine out of the second set of ten experiments, randomization and coding were performed by the head of the laboratory. One experiment was coded by a member of her laboratory. For each individual experiment, percent (%) transmission was calculated as defined in Materials and Methods. Each error bar corresponds to the standard error estimated from 4 OD values of exposed cell-tubes.

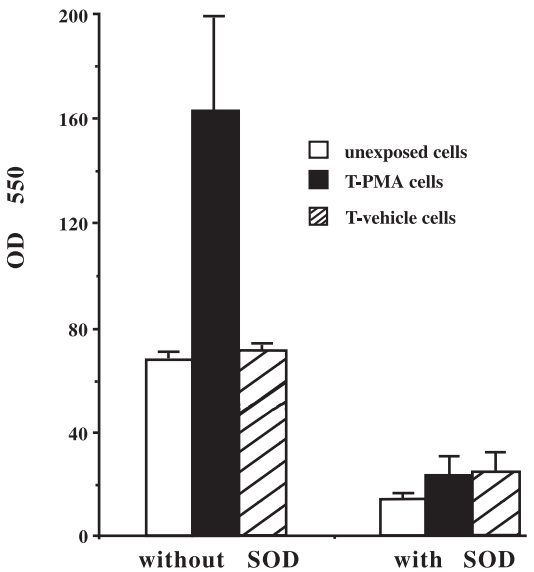

Fig. 2 SOD inhibition of the effect of T-PMA on neutrophil ROM production. Cells (1×10^6) in the absence or presence of SOD (40 μg/ml) were exposed at 37°C to either 'transmitted' PMA or 'transmitted' vehicle for 15 min. For each experiment, four transmissions were performed simultaneously (two T-PMA and two T-vehicle). In each transmission, duplicate, with versus without SOD, cell-containing target tubes were placed side by side on the output coil. As an additional control, cells were also placed 20 cm away from the output coil ('unexposed cells'). Incubation was continued for 45 min at 37°C before assessment of ROM production as described in Materials and Methods. Data are mean OD values ± S.E.M. of three independent experiments. For the sake of simplicity, integer OD values were obtained by multiplying the measured absorbance values by 1000.

T-PMA but not T-PDD stimulated ROM production suggested the involvement of PKC. To explore this possibility, we investigated the effect of two PKC-inhibitors on neutrophil activation by T-PMA. As shown in Figure 3, cells treated with GF109203X (8 μM) or H-7 (20 μM) prior to PMA transmission were less effective at cytochrome c reduction than untreated cells. GF109203X and H-7 did not affect cell viability.

A statistical summary is presented in Table 1 and Figure 4. At 60 min, T-PMA cells were associated with a 33.6 ± 3.4 % OD increase, in contrast to 2.3 ± 1.3 % ($n = 58$ transmissions, $P < 10^{-3}$, Student's *t*-test) for T-vehicle, T-PDD and oscillator power off (T-PMA off). The PMA transmission effect is not only statistically different from other groups but it is also larger by a factor of at least five (99% confidence level). The overall result is highly significant even when calculated using a very conservative, binomial approach. In 56 of the 58 binary comparisons, mean OD values for T-PMA were above those obtained for T-vehicle, T-PDD or oscillator power-off ($P = 10^{-14}$). Note also that the OD variance for T-PMA cells is higher than for T-vehicle or other exposed cells, both within individual experiments ($P = 2 \times 10^{-3}$) and between experiments ($P = 10^{-2}$). The latter data constitute additional evidence for the reality of the difference between T-PMA and other groups.

Table 2 Effect of transmitting PDD on neutrophil ROM production

Culture time length (min)	Cells exposed to	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6
15	None	ND	ND	50*	24	110	127
	T-vehicle			43	25	110	136
	T-PDD			53	24	110	144
	T-PMA			**91**	**40**	**142**	**184**
30	None	59	22	107	63	221	177
	T-vehicle	68	24	78	54	240	168
	T-PDD	67	24	88	63	220	182
	T-PMA	**78**	**70**	**107**	**90**	**245**	**232**
60	None	128	57	199	169	324	244
	T-vehicle	121	60	190	179	296	258
	T-PDD	133	56	181	173	289	258
	T-PMA	**155**	**106**	**208**	**239**	**318**	**295**

For each experiment, three transmissions were performed simultaneously. Cells (1×10^6) were exposed at 37°C to either 'transmitted' PMA (T-PMA, using a source tube containing 10 ml of PMA at 1 μM), 'transmitted' PDD (T-PDD, using a source tube containing 10 ml of PDD at 1 μM) or 'transmitted' vehicle (T-vehicle) for 15 min. As an additional control, cells were placed 20 cm away from the output coil ('unexposed cells'). Incubation was continued for an additional period (0, 15 or 45 min) at 37°C and ROM production was then assayed.

* Numbers are means of duplicate OD values. For the sake of simplicity, integer OD values were obtained by multiplying the measured absorbance values by 1000 and rounding to the nearest unit.

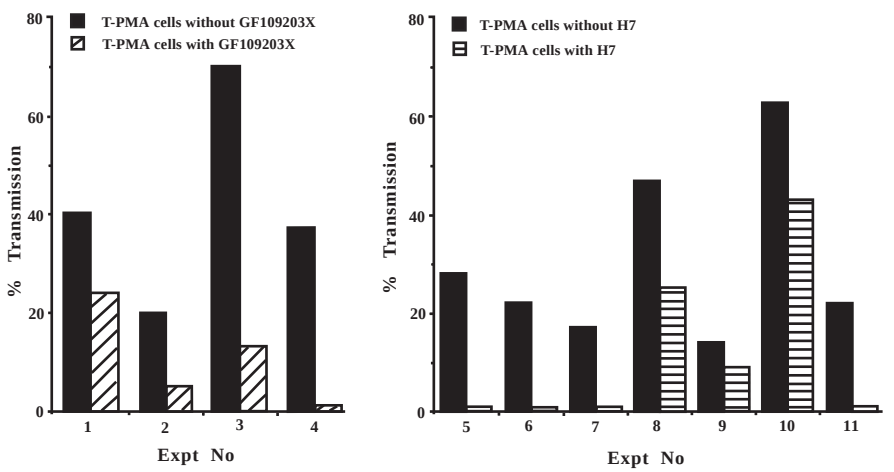

Fig. 3 Inhibition of T-PMA -induced neutrophil ROM production using PKC inhibitors. Cells (1×10^6) in the absence or presence of GF109203X, 8 μM (left panel) or H-7, 20 μM (right panel) were exposed at 37% C to either 'transmitted' PMA or vehicle for 15 min. In each transmission, duplicate (Experiments 1–4) with versus without GF109203X or triplicate (Experiments 5–11) with versus without H-7, cell-containing target tubes were placed side by side on the output coil. Incubation was continued for 15 min at 37°C and then ROM production was assessed as described in Materials and Methods. Percent (%) transmission was calculated for experiments 1–2 as defined in Materials and Methods and for experiments 3–11 with respect to T-vehicle cells.

DISCUSSION

The present work shows that normal human neutrophils reacted to PMA transmitted via an electronic oscillator by reducing cytochrome c as though they had been directly exposed to PMA. This phenomenon was not observed when the oscillator was turned off. The effect of transmitted PMA was roughly equivalent to that of 0.1 nM PMA (data not shown). In 20 experiments in which transmitted PMA was compared with transmitted vehicle, the four source tubes (two PMA and two vehicle) were randomized and blinded before each experiment. Therefore, the effect observed cannot be attributed to uncontrolled systematic factors (operator bias, temperature, time …) but to the independent variables: the contents of the source tubes and the oscillator. Similar preliminary results on the same model have been obtained, using our oscillator, by Professor W. Hsueh, Department of Pathology, Northwestern University, Chicago (personal communication).

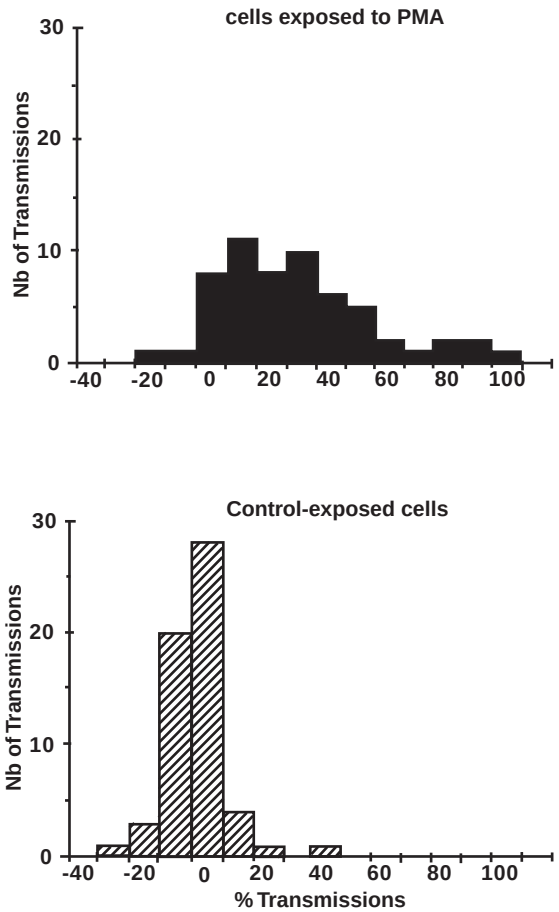

Fig. 4 Distribution of percent transmission. *Panel A*: cells exposed to T-PMA, 33.6 ± 3.4%, *n* = 58 transmissions; *Panel B*: control-exposed cells, 2.3 ± 1.3%, *n* = 58 transmissions (T-vehicle, *n* = 40; T-PDD, *n* = 6; T-PMA off, *n* = 12).

Several lines of evidence suggest that PMA transmission is a meaningful concept and that the change in OD_{550} values it provokes, biologically relevant: (1) cytochrome c reduction was dependent on the presence of cells; (2) no alteration of cell viability was induced by transmission; (3) vehicle transmission was virtually without effect. Thus, the observed transmitted PMA effect was not a nonspecific effect due to the oscillator itself or alterations of physical properties of cell components such as membranes or cytoskeleton–membrane interactions; (4) the cytochrome c reduciton observed here is, as in the case of ponderal PMA, an index of superoxide and ROM production, as it was inhibited by SOD. Finally, not only the functional resemblance to directly present PMA but the specificity of the putative transmitted PMA signal is supported by the fact that 4-α-PDD, an inactive PMA analogue transmitted in the same manner as PMA, failed to stimulate ROM production. In addition, the impact of transmitted PMA was substantially reduced in cells treated with two inhibitors of PKC, the principal cellular target of PMA.

The concept of electronic transmission of an agonist to cells is methodologically and statistically validated by the present work. Although the precise physical mechanism(s) involved remain(s) unknown, it is most likely that the PMA signal is carried as electromagnetic radiation since the material properties of the oscillator seem to exclude every other physical means of information transfer. Along this line are recent studies showing transmission of thyroxine signal via electronic circuit using water as target for the transmitted signal (14). Among the various theoretical problems associated with such a signal, two appear particularly pertinent. The first relates to background noise. Given the level of electromagnetic noise generated by the oscillator and present in the environment, it is necessary to postulate ways in which the signal-to-noise ratio or the detection of specific signals, or both, are enhanced. In fact, an appropriate level of noise enhances a specific periodic signal rather than overwhelming it, a phenomenon known as stochastic resonance (15,16). The relevance of this concept to the phenomenon reported here remains to be determined. Secondly, the limitations of the equipment used here, suggest that the signal is carried by frequencies in the low kilohertz range, many orders of magnitude below those generally associated with molecular spectra. The 'beat frequency' phenomenon may explain this discrepancy, since a detector, here the PMA receptor, will 'see' the sum of the components of a given complex wave (17).

Such preliminary and no doubt precocious speculations have little intrinsic value but serve to orient our efforts to identify the spectral nature of the signal carried from the oscillator to the neutrophils. Since we have recently recorded and digitized molecular signals whose biological impact is known, we are now analyzing thes in collaboration with a specialist laboratory. However, given the low signal-to-noise ratio, results cannot be expected in the near future.

In conclusion, the present results show clearly that, when a source tube containing PMA is placed on a coil connected to an appropriate oscillator, neutrophils in a test tube on another coil some meters away but connected to the same oscillator, produce superoxide. This effect is absent when an inactive analogue of PMA is present in the source tube. Clearly, we are presently unable to provide any theoretical explanation for these observations. Given the fruitfulness of the confrontation between theory and observation, we welcome multidisciplinarity collaboration in order to elaborate the nature of the phenomenon we have observed.

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