Biophysics-based critique of the assisted discharge mechanism hypothesis

Julie V. Stern¹, Thiruvallur R. Gowrishankar¹, Kyle C. Smith¹, and James C. Weaver¹*:¹

¹Harvard-MIT Division of Health Sciences and Technology, Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA;

*Corresponding author

Cell experiments with large, short electric field pulses of opposite polarity reveal a remarkable phenomenon: Bipolar cancellation (BPC). Typical defining experiments involve quantitative observation of tracer molecule influx at times of order 100 s post pulsing. Gowrishankar et al. BBRC 2018 503:1194-1199 shows that long-lived pores and altered partitioning or hindrance due to inserted occluding molecules can account for BPC. In stark contrast, the Assisted Discharge (AD) hypothesis, Pakhomov et al. CellMol-LifeSci 2014 71(22):4431-4441; Fig. 6, only involves early times of a microsecond down to nanoseconds. Further, well established terminology for cell membrane discharge relates to membrane potential decays shortly after pulsing. Discharge is silent on molecular or ionic transport, and does not address the fact that tracer molecule uptake vs time is measure at about 100s after pulsing ceases. Our critique of AD notes that there can be an association of AD with BPC, but associations are only necessary, not sufficient. A BPC mechanism hypothesis must be shown to be causal, able to describe time-dependent molecular influx. The two hypotheses involve very different time-scales (less than a microsecond vs 100 s) and very different quantities (volts/s vs molecules/s). Unlike pore-based hypotheses the AD hypothesis lacks explicit molecular transport mechanisms, and does not address the greatly delayed measured molecular uptake. We conclude that AD is an implausible candidate for explaining BPC.

Basic approach: Use general features of established science for plausibility estimates. This biophysics/physics method has often been used to obtain rough estimates of the plausibility of reported results, new concepts, theoretical constructs, etc. Testing is widely used, and can be extended to biophysics, including bioelectrics. In the present (biophysics of bioelectrics) order of magnitude (OOM) estimates can be compelling.

Four examples of order of magnitude estimates of plausibility using generally accepted science. These are:

(1) Implausibility of small 50-60 Hz fields causing cancer [1],

(2) Plausibility of animal sensing of very small electric fields [2],

(3) Plausibility of biological detection of small chemical reaction rates [3], and

(4) Plausibility of nsPEF causing ~100 to 1000-fold more pores than conventional electroporation [4].

Here we argue that AD (Assisted Discharge) is an implausible mechanism of bipolar cancellation (BPC). Our critique does not presently apply to excitable cells, as it considers only what is generally known about electroporation (EP), or nanopores, in non-excitiable cells. We consider generally accepted science found in the EP
Figure 1: “Bipolar pulses may assist cell membrane discharge and reduce the membrane time above the critical voltage. Cell membrane is charged (bottom) by a monopolar pulse (a, top) or a bipolar pulse (b, top). The membrane voltage (arbitrary units) goes from the baseline (solid line) to the critical electroporation voltage (dashed line) and above it. The time when the membrane voltage exceeds the critical level is shown by shading. The bipolar pulse reduces this time but does not bring the voltage below the negative critical value. See text for details.” This figure is Figure 6 from [5].

literature. We purposefully do not include experiments carried out by MURI investigators, before and during the MURI funding. The rationale is simple and basic: We want to understand what can be expected given established science, mainly publications in the biophysics literature before BPC was considered. Accepted science does not change just because someone wants to assume that BPC observed at ∼100 s is part of AD. “Assisted discharge” means discharge is faster (assisted) because of very large nsPEF fields that make so many pores that the membrane conductance is greatly increased. And that increased conductance is the cause of the more rapid (assisted) discharge through the heavily porated regions of the cell membrane. It has nothing to do with tracer molecule data and observations that occur much later, viz. at ∼100 s. Here the established scientific facts are:

(1) What is generally known about passive and porated cell membrane charging and discharging [6][7][8]. See Fig. 2, with both passive (150 mV/cm) and heavily porated (24 kV/cm) examples.

(2) What is known broadly about nanoporation due to nsPEF. Here a number of quantitative models that are broadly consistent with experiments are considered [9][10][11][12][13][14][15][16][17][18][19]. The general, established fact is that many small pores (nanopores) are created, which creates large membrane conductances.

Basic background and key definitions for BPC. A defining feature of BPC is experimentally measured decrease in the ratio of tracer molecule influx into a cell for a bipolar pulse (BP) compared to a unipolar pulse (UP). Electric field pulses are followed by cell membrane electrical discharge within a few microseconds or less. In sharp contrast, tracer influx occurs over a long time (1 - 100 s) after the pulses. This is not trivial: after the proposed important discharge, the measured effect is seen 6-8 orders of magnitude displaced in time. This means the electrically initiated molecule (tracer) transport is an exasperatingly slow process. What can account for this?! How can an early electrical event (discharge) lead to a greatly delayed tracer influx that defines BPC? This essential basic feature is missing. Further, for both UP and BP 24 kV/cm there is immediate poration. A non-porated (passive) membrane is not involved, inconsistent with Fig. 6 of Pakhomov et al. 2014 [5].
Figure 2: Response of small and large amplitude electric fields. These results show that assisted discharge only occurs for a passive membrane with negligible conductance change (zero electroporation; low strength electric field small channel conductance). The 150 mV/cm, 200 ns unipolar pulse (top left) creates 26 pores in contrast to a 24 kV/cm, 200 ns unipolar pulse (bottom left) that creates $1.6 \times 10^6$ pores in a 5 $\mu$m radius cell. As in Fig. 6 of Pakhomov 2014 [5], the bipolar case has a much faster discharge after the first half of the BP pulse. However, this occurs only for small electric fields that cause negligible electroporation. But to elicit BPC large fields (>10 kV/cm) are used, it is a contradiction. Response of two unipolar pulses (with no gap) for the 150 mV/cm and 24 kV/cm fields are shown in the right column. In all cases pores are created early (rapidly) on the first pulse and the large conduction is “remembered”.

For perspective, passive (normal) discharge time constant of a cell is about 100 ns to 1 $\mu$s for typical mammalian cells [6, 7, 8]. Electroporated cells have spatially distributed pores of various lifetimes, so the effective, local conductivity varies spatially and temporally, a complication that is readily addressed by using integrated cell system models [9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19].

For the nsPEF (nanosecond pulsed electric fields) needed to elicit BPC there is an additional feature: As shown by the Schoenbach-MURI (ODU) [4] a broad finding is that nsPEF with field strengths of order 10-100 kV/cm and sufficiently short pulses (of order 2-1,000 ns) approximately 100-1,000 more pores are created, not limited to the outer, plasma membrane (PM), but pores are also created in many organelles within the cell, including bacterial-size mitochondria with double membranes. This means that the discharge times are much more rapid. All of the above is a condensed version of what is now established nsPEF biophysics.

To our knowledge the Assisted Discharge (AD) mechanism hypothesis for bipolar cancellation (BPC) was introduced by Pakhomov and co-workers, see their Fig. 6 and associated text, included here as Fig. 1, but it has
no equations [5]. Subsequent publications cite the AD hypothesis. We find six significant flaws, identified and defined below.

Flaw #1: There is no quantitative description or prediction of tracer molecule influx, the key measured biophysical quantity that defines BPC. For an illustration of what is needed for a physics-based theory or model we cite “what Fermi told Dyson”, a one-page commentary in Nature [20]. Here Fermi states: “One way, and this is the way I prefer, is to have a clear physical picture of the process that you are calculating. The other way is to have a precise and self-consistent mathematical formalism. You have neither.” The AD hypothesis provides a qualitative picture (Fig. 1; Fig. 6 from [5]), but there are no mathematical calculations that quantify tracer molecule entry into a cell long after a pulse and its associated electrical discharge (normal or “assisted”).

Figure 3: Passive and Standard EP model response of an isolated cell model in response to a 10 kV/cm, 5 ns rise/fall time, 60 ns unipolar pulse (left) and a 60 ns + 60 ns 10/-10 kV/cm bipolar pulse (right). The passive model response is shown in solid line and the EP model response is shown by the dashed curve. The passive model response for a unipolar pulse shows the transmembrane at the pole increasing to over 4 V (for illustration only) and discharging with a time constant of around 100 ns. However, when EP is included, the response shows a reversible electrical breakdown (REB): a rapid increase in transmembrane voltage followed by a decrease to a plateau which rapidly reaches zero after the pulse ends (<1 ns). In the case of the bipolar pulse, the transmembrane voltage decreases much faster (along the lines of assisted discharge) to a smaller negative peak before decaying to zero with a time constant of around 100 ns. With the inclusion of EP, there is no REB peak during the second half of the BP. Instead the transmembrane voltage remains around -0.44 V. REB is characterized by a rapid increase in transmembrane voltage at the onset of a pulse which leads to a burst of pore creation leading to a large increase in membrane conductance. This REB increase in conductance brings down the transmembrane voltage magnitude, limiting pore creation even before the pulse ends. REB is seen as a plateau in transmembrane voltage following a transient spike. As the unipolar pulse response (left) shows that without EP, post-pulse transmembrane voltage decays with a time constant of 112 ns while significant EP causes the transmembrane voltage to decrease much faster with a time constant of 10 ns.

Flaw #2: There is no electroporation “critical value”, but instead measurement thresholds. There is no phase-transition type behavior with a rather sudden change, here involving increasing $\Delta \phi_m$ (membrane potential or transmembrane voltage). The horizontal dashed line(s) in Fig. 6 in [5] are detection or measurement thresholds that depend on both pore creation rates at different cell membrane locations, and also on experimental measurement capabilities. There is nothing “critical” about these events. Instead they are measurement thresholds for either actual or Gedanken experiments, governed by signal-to-noise ratio (S/N). Continuum theories and molecular dynamics
simulations are consistent regarding the conditions needed to observe effects due to significant poration for particular experimental conditions (cell geometry and size, pulse waveform, etc.). Detection (measurement) theory is well known in physics (and therefore biophysics involving bioelectrics) [1, 3, 21, 22, 23].

Flaw #3: Orders of magnitude different time scales. For typical mammalian cells passive discharge occurs over times of 100 nanoseconds to a few microseconds; active discharge due to applied fields can be much faster if the field creates many pores (e.g. nsPEF). But influx of tracer molecules occurs long afterwards, with tracer influx time scales of 1 to 100 seconds. The AD mechanism offers no explanation for this huge discrepancy.

Flaw #4: Cell models must be 2D or 3D, with both an “inside” and “outside”. This feature is needed to account for field and ion/molecule transport both “around” and “through” the porated plasma membrane (PM). A cell model cannot be represented by a local planar membrane model. This is widely recognized in the literature [19, 9, 10, 11, 12, 13, 14, 15, 16, 18, 19].

Flaw #5: Small field pulses should yield Schwann (passive) model behavior. Pulses too small to create nanopores should automatically revert to well-known passive membrane behavior (Fig. 2) [6, 7, 8]. It is not enough to explain BPC. The model must also be consistent with all other relevant biophysical behavior, e.g. response of cells to pulses too small to create large permeabilities or conductances in cell membranes. Passive model responses do not account for the many orders of magnitude increase in membrane conductance and the resulting drop in transmembrane voltage magnitude (Fig. 2).

Flaw #6: AD occurs mainly for non-porating fields (Figs. 2, 3). But, fields that elicit BPC create over $10^5$ pores, a very large number. These pores cause a much faster membrane discharge ($\sim 10$ ns) after the pulse.

In general, the literature contains lots of results for pore behavior after pore creation. This undercuts the idea that one should avoid treating what happens above the critical membrane potential, which is the implication of the AD mechanism hypothesis.

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