Electrophysiology of the phagocyte respiratory burst. Focus on "Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity"

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Electrophysiology of the phagocyte respiratory burst. Focus on "Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity"

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THERE IS A TREND, WHICH SEEMS quite reasonable to me, toward giving papers titles that are declarative summaries of the main conclusion of the paper, in contrast to traditional titles, which simply describe the topic studied. A busy (or lazy) reader can extract the gist of the story without even reading the abstract. One danger of the "conclusion-as-title" approach is that if the conclusion is wrong, the title may become embarrassing. Such is the case with "The large conductance Ca^{2+}-activated K^+ channel is essential for innate immunity," by Ahluwalia et al. (1). The fundamental elements of this large-conductance Ca^{2+}-activated K^+ (BK) channel hypothesis of innate immunity are as follows: *1*) BK channels (also known as *slo* or maxi-K^+ channels, which are activated by elevated intracellular Ca^{2+} concentration and membrane depolarization) comprise the main conductance in activated human neutrophils and eosinophils, and *2*) BK channel inhibition prevents the killing of bacteria and fungi by neutrophils. The goal of the study by Essin and colleagues (7) was to evaluate this hypothesis; their results unambiguously contradict it. They refute the sine qua non of the hypothesis by showing that BK channels are not expressed in human or mouse neutrophils. No BK current was detected in neutrophils under conditions that elicit BK current in smooth muscle or when solutions identical to those used by Ahluwalia et al. were used. The study of Essin and colleagues complements and extends a recent study that arrived at similar conclusions (8).

Although phagocytes (neutrophils, macrophages, and eosinophils) are officially nonexcitable cells, they have an electrophysiology that is at least as rich as that of muscle or nerve cells (5). Macrophages seem never to have met a channel they did not want to express, at least under certain circumstances (9). Neutrophils and eosinophils are more discriminating, expressing fewer, yet exotic, membrane transporters (e.g., electron and proton pathways). A major weapon used by phagocytes in their unceasing efforts to protect us from extracorporeal invaders is the NADPH oxidase complex, which produces superoxide anion ($O_2^{\bullet -}$), the precursor to a variety of reactive oxygen species that help kill microbes (13, 16). The NADPH oxidase complex is electrogenic: it moves electrons across the membrane, from intracellular NADPH to the extracellular or intraphagosomal space, where O_2 is reduced to $O_2^{\bullet -}$ (11). The prevailing view is that voltage-gated proton channels open to allow H^+ efflux, which balances the loss of negative charge due to electron efflux (6, 11, 17). In contrast, Ahluwalia et al. (1) identified the outward conductance of neutrophils and eosinophils as due to BK channels, which were touted as essential to the primary function of neutrophils, i.e., killing bacteria.

The study by Essin et al. (7) is not the first crack in the facade of the BK channel hypothesis. From the outset, a number of the results of Ahluwalia et al. (1) contradicted a sizable body of data in the literature (summarized in Ref. 4). *1*) No previous study had identified BK channels in neutrophils or eosinophils. *2*) The magnitude of BK currents reported was at least an order of magnitude larger than any conductance observed in previous studies. *3*) Addition of Zn^{2+} to activated cells had no effect, suggesting a lack of proton currents, in contrast to observations reported in numerous studies. *4*) Phagocytes undergo a massive and sustained depolarization during NADPH oxidase activity (2, 10, 12, 15). If a K^+ conductance of the magnitude reported for BK channels were active in these cells, the electron current generated by NADPH oxidase could not depolarize the membrane potential by more than a few millivolts above the Nernst potential for K^+, an intuition confirmed by quantitative modeling (14).

A number of laboratories around the world, independently or in concert, have conducted a variety of studies in response to the report of Ahluwalia et al. (1). The BK channel hypothesis was debated vigorously at the Phagocytes Gordon Research Conference in June 2005 and at the European Society of Clinical Investigation Phagocyte Workshop in Prague in March 2006. Until the study of Essin et al. (7), however, only one paper had been published, and that was only after struggles with referees and editors of several journals. More than two years after the publication of Ahluwalia et al. in 2004, we published a systematic study (8) that evaluated the BK channel hypothesis and unequivocally contradicted every major result of their paper. Our electrophysiological studies revealed a complete absence of BK channels in human neutrophils and eosinophils, the cells studied by Ahluwalia and colleagues. This result was echoed by the absence of BK channel protein in Western blots of neutrophil membranes. BK channel inhibitors had no effect on any component of current in these cells under a variety of conditions designed to favor BK channel opening and also to mimic conditions in intact cells during the respiratory burst. Finally, BK channel inhibitors had no effect on any of several key functional activities of neutrophils: $O_2^{\bullet -}$ production, NADPH oxidase-dependent degradation of bacterial phospholipids, and, most importantly, bacterial killing.

Given this resounding refutation of the BK channel hypothesis, why is the study by Essin et al. (7) so important? It is a fact of scientific life that a high-profile publication often carries far more weight than it should. A related truism is that when two studies arrive at different conclusions, most readers feel technically unable to evaluate which is correct and simply conclude that the subject is controversial and that the correct result will be established at some point in the distant future.

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This attitude is not unreasonable: it is very difficult to evaluate technical details of methods one does not use routinely. In the interest of advancing knowledge, then, it is essential to publish multiple studies to correct mistakes that appear in the literature, particularly in prominent places. It is significant, then, that the crucial tests of the effects of BK channel inhibitors on the killing of bacteria and fungi by neutrophils were conducted independently in laboratories in Berlin and Tübingen. In our study (8), bacterial killing was tested independently in the laboratories of Drs. William M. Nauseef (University of Iowa) and Erzsébet Ligeti (Semmelweis University, Budapest). One subtle difference in the killing assays is that in the study of Ahluwalia et al. (1) the bacteria were washed after they were treated with purified IgG, but our colleagues did not wash the bacteria after serum opsonization. Conceivably, serum present in the killing assay might bind the BK channel-inhibitory toxins. However, Dr. Ligeti's group examined this possibility (personal communication) and found that the presence or absence of serum (i.e., washing or not washing the opsonized bacteria) had no effect on the killing of bacteria or the ineffectiveness of iberiotoxin. At the Workshop in Prague, Dr. Eva Decleva (University of Trieste, Trieste, Italy) reported bacterial killing assays that indicated no effect of BK channel inhibitors on the killing of *Candida albicans* or *Staphylococcus aureus* by human neutrophils (3). In unpublished experiments (personal communication), Drs. Dirk Roos and Anton Tool (Sanquin Research at CLB, Amsterdam, The Netherlands) found no effect of the BK channel inhibitors iberiotoxin or paxillin at concentrations used by Ahluwalia et al. (1) on the survival of *S. aureus* type O or *Escherichia coli* in the presence of human neutrophils or on the perforation of *E. coli*, assessed by availability of the bacterial enzyme β-galactosidase for its substrate. In total, using a variety of approaches and assays, at least seven laboratories worldwide have independently attempted to reproduce the report that BK channel inhibitors prevent human neutrophils from killing microbes, and each found no effect.

The study by Essin et al. (7) does more than just double the number of published papers that definitively refute the BK channel hypothesis. Essin and colleagues extend previous work in two important respects. *1*) They show that *N*-formyl-L-methionyl-L-leucyl-phenylalanine, a physiological agonist that acts by increasing intracellular Ca^{2+} concentration, does not elicit detectable BK current. If there were a situation in neutrophils in which BK channels should be activated, it is in the *N*-formyl-L-methionyl-L-leucyl-phenylalanine (fMetLeuPhe) response, when the intracellular Ca^{2+} concentration increases and the membrane potential depolarizes drastically (15). *2*) Essin et al. (7) use BK channel-knockout mice to evaluate the putative role of BK channels in phagocyte function. The electrophysiological responses of the $BK^{+/+}$ murine phagocytes were identical to those of the $BK^{-/-}$ cells, indicating prominent proton currents and a lack of BK currents. In addition, in mouse infection models, there was no difference in bacterial or fungal clearance from the control and knockout mice. Thus, Essin and colleagues have added a direct, in vivo evaluation of the hypothesis that is free from any pharmacological concerns. It is now difficult to think of a definitive test of the BK channel hypothesis that has not been done. All the tests give the same result.

This case is not an indictment of the peer review process, although the reviewers should have demanded more rigorous proof of the existence of BK channels (namely, measurements of the reversal potential at various K^+ concentrations and positive controls on the immunoblot). However, when a reviewer is presented data indicating that BK channel inhibitors prevent neutrophils from performing their main function, how can he/she know whether this result will be reproducible? A great deal of time, energy, and expense has been consumed by the efforts of many laboratories to evaluate what can only be described as an erroneous hypothesis. Is there any alternative? Should journals ask authors to replicate their own studies if major components cannot be reproduced by other laboratories? Should wrong results be retracted more often than they are? Or does the truth come out eventually, in any case? I hope that the study of Essin et al. (7) is the final episode: that no more studies will be required to put the BK channel hypothesis to rest.

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