We read with great interest the article by Bingen et al. 1 entitled ‘Prolongation of minimal action potential duration in sustained fibrillation decreases complexity by transient destabilization’. This paper referred to our report that 2-aminoethoxydiphenyl borate (2APB) induces fibrillation in perfused rat hearts 2 (http://youtu.be/pDsM0UKQv34, http://youtu.be/fjQ_yfPzyBwk). It also extended our work 3–7 and that of Huo et al. 8 by establishing the arrhythmogenicity of 2APB in monolayers of neonatal rat ventricular myocytes. Bingen proposed that 2APB provokes electrical instability by inhibiting cardiac connexins which leads to impulse re-entry. They also concluded that prolonging the action potential with Bay K 8644 or barium reduces ectopic complexity to a few re-entrant sources. We would like to highlight four points that are inconsistent with these analyses and suggest an alternate, possibly complementary, explanation for 2APB arrhythmia. 7

First, the concentrations of 2APB that initiate sporadic ectopy (≏10 μM) 1,10 do not affect connexin 43 and 45. 9 Furthermore, 15–20 μM 2APB which induces tachycardia and fibrillation 12 should not appreciably affect connexin 43 while reducing connexin 45 activity about one-half. 9 Transgenesis shows that only large decreases in connexin activity provoke re-entrant arrhythmia. 10 Since arrhythmogenic concentrations of 2APB directly affect connexins to a limited degree, it is not clear how they would so decrease conduction velocity and provoke re-entry. 1 Electromechanical uncouplers and voltage-sensitive dyes themselves decrease impulse conduction. 11,12 This may contribute to the low conduction Bingen reports at doses of 2APB that do not greatly affect connexins. 

Secondly, Bingen’s voltage-mapping studies expand our data which demonstrated that Bay K 8644 induces organized, high-frequency ectopy in left atria or left ventricular papillary muscles treated with 2APB. 4,5,6 While Bingen concluded that the prolongation of the action potential duration underlies this effect, FPL-64176, isoproterenol, and ouabain also provoke high-frequency ectopy to similar extents. 4 FPL-64176 prolongs the action potential much more than Bay K 8644. 13 Isoproterenol increases this variable to a similar degree as Bay K, 14 whereas ouabain reduces action potential duration. 15 Thus it would be of interest to test if action potential duration correlates with re-entrant complexity in myocyte cultures treated with 2APB and exposed to this panel of compounds.

Thirdly, small molecules such as SKF-96365 and ML-7 suppress the sporadic ectopy, the high-frequency ectopy, and the fibrillation that 2APB induces in heart muscle. 2,5,6,7 They also interdict voltage-independent calcium signalling. 14 In addition, calmodulin antagonists but not calmodulin-dependent protein kinase II inhibitors suppress 2APB ectopy. 2,5,6 Our recent data show that bcl-2 inhibitors similarly stifle 2APB ectopy. 7 It would be useful to test if these diverse molecules increase impulse conduction in cultures treated with 2APB or in untreated monolayers, as the restoration of impulse conduction would be required to suppress 2APB ectopy or re-entry were its underlying cause.

Fourthly, 2APB provokes electromechanical ectopy even when it is added to quiescent non-automatic left atria or papillary muscles (e.g. Figure 3B: * and ‡ in ref. 6). Since re-entry requires a preceding impulse, how this mechanism for arrhythmia might induce the initial spontaneous depolarization of unpaced muscles treated with 2APB is not apparent to us. Understanding the origin of these primary events which are by definition non-re-entrant will help dissect the molecular mechanism underlying 2APB arrhythmia.

Towards this goal, it is known that 2APB stimulates voltage-independent calcium entry in non-excitable cells through the Orai calcium channels with EC50 identical to those that cause cardiac ectopy. 17 These channels, and the related transient receptor potential proteins, are important regulators of cell calcium signalling. Huo published that 2APB activates calcium entry in isolated myocytes 6; we reported that Orai inhibitors suppress 2APB ectopy and that rat left atria and ventricles express Orai1 and Orai3. 3,5,6 Thus the activation of these channels in an excitable cell background may stimulate a novel calcium-linked pathway for automatic arrhythmia, a notion which we have outlined elsewhere in more detail 3,5,7.

There are at least three ways that this novel mechanism may be important in arrhythmia. Firstly, intra- and extra-cellular signals including calcium store depletion and inflammatory mediators provoke calcium entry through voltage-independent calcium channels. Thus these channels may provide an unexpected way to couple well-known pathophysiological stimuli to arrhythmia. 8 Secondly, calmodulin-dependent protein kinase II participates in triggered afterdepolarization. 19,20 If our alternate mechanism were to initiate focal atypical automaticity, then calcium signalling could underlie both the triggered and the automatic events that lead to re-entry. The third, the two suggested mechanisms for 2APB ectopy, connexin impairment, 1 and aberrant voltage-independent calcium signalling, 2,7 may not be mutually exclusive. That is, calcium entry through voltage-independent calcium channels may offer an unexpected means to suppress connexin activity. Further study of 2APB ectopy may reveal new mechanisms for arrhythmia and identify unforeseen therapeutic
device.
Prolongation of minimal action potential duration in sustained fibrillation decreases complexity by transient destabilization: reply

In their letter, Wolkowicz et al. raise a valid point questioning whether other effects of 2-aminoethoxydiphenyl borate (2-APB) might also have contributed to ventricular fibrillation (VF) initiation, especially its effects on voltage-independent calcium channels, as was also mentioned in the Discussion section of our manuscript recently published in Cardiovascular Research.

In this study, we showed that prolonging the minimal action potential duration (APD) could decrease the complexity (e.g. number of rotors) during sustained VF. To induce fibrillation in neonatal rat ventricular cardiomyocyte monolayers and in adult rat hearts, we used 2-APB. We postulated that the re-entry conduction patterns resembling VF after 2-APB treatment could be partly attributed to the effect of 2-APB on gap junctional coupling.

The main purpose of our study was to study the maintenance properties of VF. Going into length on the mechanism by which 2-APB induces VF would defeat this purpose, as VF can show the same maintenance properties regardless of how it is initiated. Hence, we fully agree with Wolkowicz et al. that 2-APB might contribute to VF initiation by other means than only gap junctional uncoupling. Considering that virtually all pharmacological agents are aspecific to some extent it can be expected that different, potentially interacting factors may be responsible for a certain effect.

In our study, we showed a significant decrease in conduction velocity after treatment with 2-APB. Wolkowicz et al. suggest that this decrease might have been caused by the use of electronic mechanisms and voltage-sensitive dyes, and that the effect of 2-APB on gap junctional communication was too little to reach a threshold for arrhythmogenesis. However, in the experiments using cardiomyocyte monolayers we did not use electronic uncoupling, while the same concentration of voltage-sensitive dye was used in control and 2-APB-treated cultures or in control and 2-APB-treated hearts. Hence, in our setup the specific effects of these pharmacological agents do not seem to provide a plausible explanation for the different in conduction velocity. Nevertheless, the decrease in gap junctional uncoupling by BDM treatment or the decrease in conduction velocity by di-4-ANEPPS could effectively lower the degree of additional uncoupling needed to reach the arrhythmogenic threshold, which could help explain the tachyarrhythmias found after 2-APB treatment.

Furthermore, we showed that in cultures with VF, prolongation of the minimal APD by BayK8644 and BaCl2 decreases the activation frequency and complexity of VF. Wolkowicz et al., however, found strongly increased activation frequencies when combining 2-APB with BayK8644 treatment. The differences in results could be attributable to the fact that different models and protocols were used. For example, in our study cells were first incubated with 2-APB for 20 min, after which they were subjected to optical mapping and BayK8644 treatment. In contrast, Wolkowicz et al. used pre-treatment with BayK8644 and direct analysis of activation frequency after the addition of 2-APB. Possibly, the mechanisms causing high activation frequency after short- or long-term 2-APB treatment are different. Wolkowicz et al. showed that the increased activation frequency is caused by abnormal automaticity, as a result an increase in APD by BayK8644 pre-treatment does not decrease the frequency. We show that after incubation for 20 min with 2-APB there is a predominant re-entrant activation pattern, in which BayK8644 decreases the maintenance properties of VF, despite the incompleteness of our data. Nevertheless, this does not exclude the possibility that before re-entry initiation there is 2-APB-induced non-re-entrant automaticity in our setup as suggested by Wolkowicz et al., which would strongly increase the chance of re-entry initiation in our cultures.

In conclusion, we acknowledge the issues Wolkowicz et al. raised in their letter and support the notion that indeed 2-APB-induced arrhythmias can be initiated by mechanisms other than gap junctional uncoupling. However, based on our data we feel that our model, in which stable re-entrant action patterns are observed after longer 2-APB incubation, allows for studies on sustained VF, despite the incomplete knowledge of its initiation. For more specific and mechanistic studies on the origin of arrhythmias genetic interventions based on viral vector technologies seem more appropriate, especially when such interventions are designed to be cell type-specific and inducible. Nevertheless, additional research is needed to better understand the mechanisms behind 2-APB-induced arrhythmias as this might contribute to the development of novel anti-arrhythmic strategies.

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