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Dimethylsulfoxide and ethanol, commonly used diluents, prevent dilation of pial arterioles by openers of K_{ATP} ion channels ☆

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Abstract

Ethanol and dimethylsulfoxide are commonly used as diluents for water-insoluble drugs. Both are antioxidants. An earlier study of cats presented pharmacological evidence indicating that oxidants could open the K_{ATP} ion channel in cerebral surface arterioles [pial arterioles] and that antioxidants including dimethylsulfoxide and L-cysteine prevented opening of these channels. Ethanol was not tested. The present study extends the older observations to a second species, the rat, and examines ethanol as well as dimethylsulfoxide and L-cysteine. A microscope and image splitter were used to measure arteriolar diameters under a closed cranial window in pentobarbital-anesthetized, paralyzed rats. Drugs were topically applied. Dose-dependent dilations produced by two well-established openers of the K_{ATP} ion channel were inhibited in dose-dependent manner by ethanol at doses from 0.01% to 0.075%. Above this dose, the effect disappeared. Dilation by sodium nitroprusside was not affected. Dimethylsulfoxide and L-cysteine inhibited dilation produced by pinacidil. Dimethylsulfoxide inhibited pinacidil in a dose-dependent manner at doses from 0.01% to 0.2%. L-Cysteine inhibited pinacidil. Since all the inhibitory drugs have antioxidant properties, their effect may be a reflection of that property as suggested in an earlier paper. Ethanol and dimethylsulfoxide inhibited in doses frequently present when these agents are used as solvents. When investigators use these solvents to dissolve water-insoluble, topically applied drugs, we suggest that they first test the possibility that their observations are being made under conditions in which opening of the K_{ATP} ion channel is inhibited.

Keywords: Ethanol; Dimethylsulfoxide; Cerebral circulation; Pial arteriole; Vasodilatation; ATP-dependent potassium channel; K_{ATP} ion channel; Pinacidil; Cromakalim; Sodium nitroprusside

1. Introduction

Ethanol and dimethylsulfoxide are commonly used as diluents for water-insoluble drugs. As such, they are often employed in studies of the responses of arterioles on the brain's surface (pial arterioles). However, in 1998, Wei et al. reported that very low concentrations of dimethylsulfoxide inhibited dilation of cat pial arterioles by pinacidil and cromakalim, established openers of the K_{ATP} ion channel (Quale et al., 1997; Faraci and Heistad, 1998). Dimethylsulfoxide is an antioxidant and was one of several antioxidants (Wei et al., 1998) that were found to inhibit dilation by K_{ATP} channel openers. Earlier pharmacological data (Wei et al., 1996) had already indicated that the open state probability of the K_{ATP} ion channel could be increased by oxidizing agents, including those generating oxygen radicals. Therefore, it was suggested that the ability of antioxidants to inhibit the action of K_{ATP} channel openers might be dependent upon their antioxidant property. Extremely low concentrations of dimethylsulfoxide were affective but no mention was made of the implications of this finding for the interpretation of studies employing dimethylsulfoxide as a diluent of water-insoluble compounds. The present study pursues the latter concern.

We first attempted to duplicate in another species, the rat, the finding (Wei et al., 1998) that dimethylsulfoxide and L-cysteine each inhibited the response to pinacidil. When this effect was, indeed, observed, we concluded that the results in cats were not dependent upon the use of that species, and we proceeded to test ethanol, another antioxidant that is commonly used as a diluent of water-insoluble compounds. Extremely dilute solutions of ethanol inhibited dilation by either pinacidil or cromakalim. The inhibitory effect disappeared at lesser dilutions [higher concentra-

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tions] of ethanol. These data, reported below, call attention to the possibility that when either dimethylsulfoxide or ethanol is used, the experimental conditions may be altered so that the ability to open the K_{ATP} ion channel is significantly reduced. Therefore, the conclusions of published studies that used these diluents may have been made, unbeknownst to their authors, under conditions that minimized the ability of the K_{ATP} ion channel to play a role in mediating the results. Future studies using one of these diluents can test this possibility by beginning with a comparison of the ability of a K_{ATP} channel opener to dilate the arterioles in the absence of the diluent with its ability to dilate the arteriole in the presence of a concentration of the diluent equal to the final concentration planned to be used in the experiment.

2. Methods

2.1. Preparation—general

Experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Male Sprague–Dawley rats (250–350 g body weight) were anesthetized with sodium pentobarbital (55 mg/kg i.v.). A femoral vein was cannulated for additional anesthetic as needed. A femoral artery was cannulated for continuous measurement of blood pressure and periodic determination of blood gas and blood pH values. After completion of a tracheotomy, each rat was ventilated on a positive pressure ventilator following paralysis by pancuronium bromide (3 mg/kg i.v.). Respirations were adjusted to maintain P_aCO_2 between 35 and 40 mm Hg and were kept constant throughout each experiment. Mean ± standard deviation (S.D.) values for the blood gases were: $P_aO_2 = 84 \pm 5$ mm Hg, $P_aCO_2 = 39 \pm 2$ mm Hg. The mean pH was 7.44 ± 0.03 and mean arterial blood pressure was 112 ± 3 mm Hg.

2.2. Cranial window

Cerebral microcirculation of the parietal cortex was observed with a Wild microscope through an acutely implanted, closed, cranial window (Levasseur et al., 1975; Ellis et al., 1983) filled with mock cerebrospinal fluid (CSF) (Raper et al., 1972). There were three outlets from the window. Two were inflow and outflow paths used only to replace the control mock CSF with mock CSF containing a drug, or to wash out such solutions to reestablish a baseline. The pH of the fluid placed under the window was adjusted to 7.35 by equilibration with a mixture of 6% O_2, 6% CO_2 and the balance N_2. Diameter of pial arterioles was measured with a Vickers image-splitting device. Usually three to six pial arterioles were measured in each rat. All monitoring took place with the mock CSF stationary under the window. The third opening in the window was used to continuously monitor the intracranial pressure that was maintained at 5 mm Hg with a fluid column also connected to this port and kept at a predetermined height.

2.3. Drugs

Dimethylsulfoxide, ethanol, L-cysteine, pinacidil, cromakalim and sodium nitroprusside were obtained from Sigma. L-Cysteine and sodium nitroprusside were dissolved in mock CSF at a final pH of 7.35. Pinacidil and cromakalim stock solutions were made in ethanol and diluted in mock CSF before use. The final pH was 7.35. The final concentration of ethanol was 0.03% for 1 μM pinacidil and 0.06% for 2 μM pinacidil. In one study, the response to pinacidil in these concentrations of ethanol was compared with the response to 1 and 2 μM pinacidil in a final concentration of 0.5% ethanol. When cromakalim was used, the final concentration of ethanol was 0.3% for 1 μM and 0.6% for 2 μM. These concentrations of ethanol had been found to have no effect on the response to cromakalim.

2.4. Experimental design

The space under the window was filled with mock CSF and the vessel diameters at resting state were measured. Three to six arterioles were monitored in each rat. The fluid under the window was replaced with mock CSF containing various drugs. Cumulative responses were obtained to 1 and 2 μM of pinacidil or cromakalim. Maximal response to each dose was the parameter of interest and was obtained with the chamber outflow sealed so there was no flow of mock CSF under the window. This response was obtained in 1–2 min. Thus, during the determination of the initial dose response to pinacidil or cromakalim, the total exposure to alcohol in the diluent was no more than 4 min. After this, the chamber was flushed with mock CSF and the vessels returned to baseline diameter. The mock CSF was then replaced with mock CSF containing either dimethylsulfoxide, ethanol, or L-cysteine at a final pH of 7.35. Only one drug was tested per rat. Various concentrations of dimethylsulfoxide, ethanol and L-cysteine were used, as indicated in Section 3. All were presented in a sealed chamber. Each concentration remained in place for 15 min. The cumulative dose response to two doses of pinacidil or cromakalim was again determined. After this, there was a 10-min washout with mock CSF with return of diameter to baseline. The next treatment dose was then tested, it remained in the sealed chamber for 15 min, followed by a new test of the response to both doses of pinacidil or cromakalim. The experiment in each rat ended with a final 10-min washout with mock CSF and a retest of the response to the two standard doses of pinacidil. In one set of experiments using

ethanol as the treatment, sodium nitroprusside was used as the dilator rather than an opener of K_{ATP} ion channels.

2.5. Statistics

Dilations were expressed as a percentage of basal diameter. Since more than one arteriole was monitored in each rat, the responses of all the arterioles in a given rat were averaged and these average values ± standard deviations were used in the statistical analysis. Thus, the "*N*" for each experiment is equal to the number of rats rather than to the much larger number of arterioles. Treatment effects were determined using analysis of variance (ANOVA). A *P* value of 0.05 was considered significant. We determined differences between groups within a significant ANOVA by using each rat as its own control and comparing pre- and posttreatment responses using the paired "*t*" test. Differences were considered significant when the *P* value was equal to or less than 0.05, but, in fact, in most cases, the differences were significant at the 0.01 level as shown in Section 3.

3. Results

3.1. Repetition in rats of results previously reported in cats

3.1.1. Effect of dimethylsulfoxide on response to pinacidil

Fig. 1 shows that dimethylsulfoxide inhibited the response to pinacidil. Inhibition increased as dose increased from 0.01% to 0.1%. There was no change in diameter during incubation with dimethylsulfoxide. Time controls showed that the inhibition of the response to pinacidil had nothing to do with the passage of an equal amount of time, the response to pinacidil being 10 ± 2% and 18 ± 5% at the beginning and 12 + 2% and 21 ± 3% at the end of the control experiments.

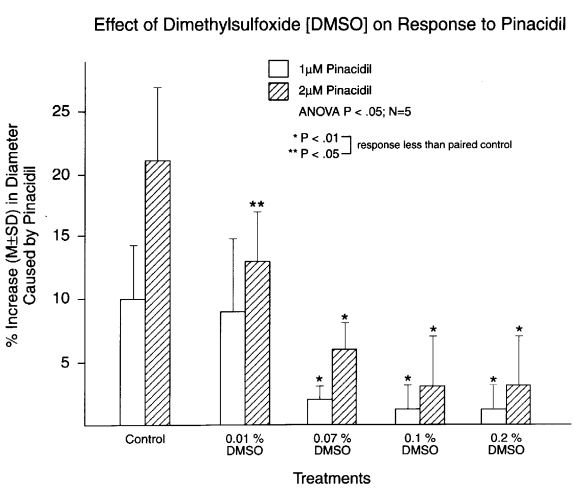

Fig. 1. Dimethylsulfoxide inhibited the dose-dependent dilation by pinacidil. Inhibition increased with ascending doses of dimethylsulfoxide. $N = 5$ rats. There were 17 arterioles with diameter 47 ± 7 μm (mean ± S.D.).

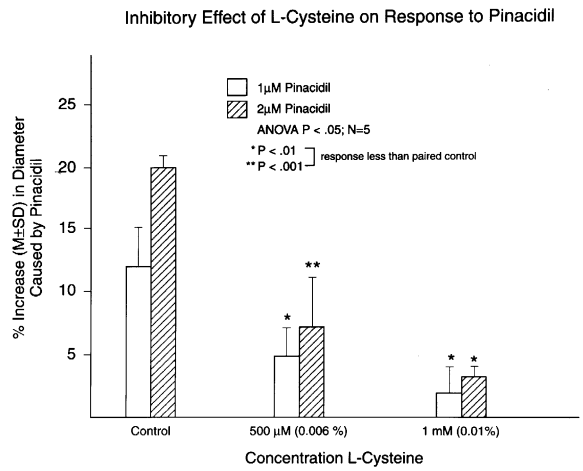

Fig. 2. L-Cysteine inhibited dose-dependent dilation by pinacidil. $N = 5$ rats. There were 20 arterioles with diameter 43 ± 4 μm (mean ± S.D.).

3.1.2. Effect of L-cysteine on response to pinacidil

Fig. 2 shows that L-cysteine 0.006% [500 μM] and 0.01% [1 μM] had successively greater inhibitory effects on the response to pinacidil. L-Cysteine itself caused no change in diameter.

3.2. New data—effect of ethanol in rats

3.2.1. Ethanol at high dilutions but not at lesser dilutions inhibits response to pinacidil

Fig. 3 shows the dose-dependent dilation produced by pinacidil and the inhibition of that response by 0.03% and 0.075% ethanol. The effect of 0.075% ethanol on the higher concentration of pinacidil was less than the effect of 0.03% and 0.5% ethanol; the highest dose of ethanol used

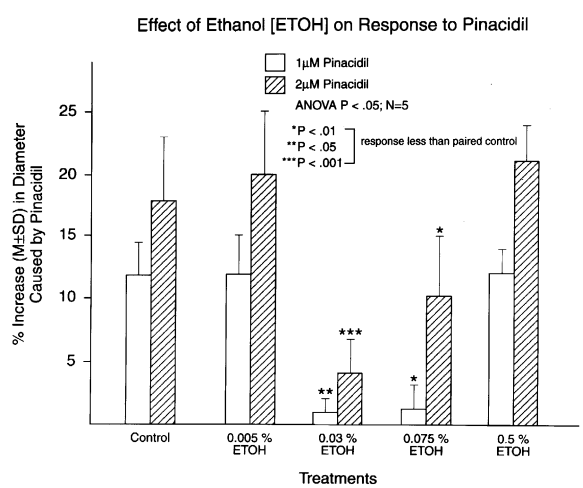

Fig. 3. Ethanol [0.03–0.075%] inhibited dose-dependent dilation by pinacidil. A higher dose of ethanol had no inhibitory effect. $N = 5$ rats. There were 18 arterioles with diameter 44 ± 3 μm (mean ± S.D.).

in this experiment had absolutely no effect on either concentration of pinacidil. An additional experiment in five more rats not displayed in a figure, showed that either 0.01% or 0.05% ethanol markedly inhibited the response to both doses of pinacidil ($P < 0.05$). Dilation (mean ± S.D.) by 1 and 2 μM was 12 ± 8% and 23 ± 5%, respectively, before treatment with ethanol and was reduced to 2 ± 1% and 4 ± 3% by 0.01% ethanol and to 1 ± 1% and 7 ± 4% by 0.05% ethanol. After treatment with 0.1% ethanol, dilation produced by low-dose pinacidil increased to 7 ± 5% and that produced by high-dose pinacidil increased to 17 ± 6%; values not significantly different from the dilation produced by pinacidil before ethanol treatment. In neither experiment did any of the doses of ethanol itself alter resting diameter. In summary, we found that ethanol in doses as low as 0.01% but not 0.005% reduced the response to pinacidil. The effects of ethanol at doses of 0.01% and 0.03% were similar and declined at higher doses, becoming statistically insignificant at 0.1% and totally absent at 0.5%.

3.2.2. Effect of ethanol on sodium nitroprusside

A control experiment showed that the effect of ethanol was selective in that it failed to alter the response to sodium nitroprusside. Here, 0.5 and 1.0 μM sodium nitroprusside elicited dilations of 18 ± 2% and 35 ± 4% before 0.01% ethanol and 20 ± 5 and 35 ± 7% after ethanol ($N = 4$ rats, 12 vessels, diameter 44 ± 5 μm).

3.2.3. Effect of ethanol used to dissolve pinacidil but without preincubation with ethanol

Since pinacidil is poorly soluble in water, stock solutions were made in ethanol and then diluted in mock CSF as explained in Section 2. When we discovered that preincubation with low concentrations but not high concentrations of ethanol, by themselves, inhibits the response to pinacidil, it became necessary to see whether the response to pinacidil was reduced when, in the absence of preincubation with ethanol, low concentrations of ethanol were present in the final dilutions of pinacidil. Therefore, we compared the response to the usual solutions of 1 and 2 μM pinacidil, containing 0.03% and 0.06% ethanol, respectively, with the response to pinacidil in 0.3% and 0.6% ethanol, concentrations which, as shown above, do not inhibit the response to pinacidil even after incubation for 15 min. The two tests of pinacidil were separated by a 10-min washout with mock CSF free of any ethanol. Thus, the responses to 1 and 2 μM pinacidil were 11 ± 3% and 19 ± 3% in the usual, lower concentration of ethanol and 14 ± 2% and 22 ± 3% in the higher concentration of ethanol; $N = 5$ rats, arteriolar diameter 39 ± 3 μm. The slightly lower responses to pinacidil in the usual, lower concentrations of ethanol were not significantly smaller than those in the greater concentration of ethanol (paired "t" test, $P > 0.05$).

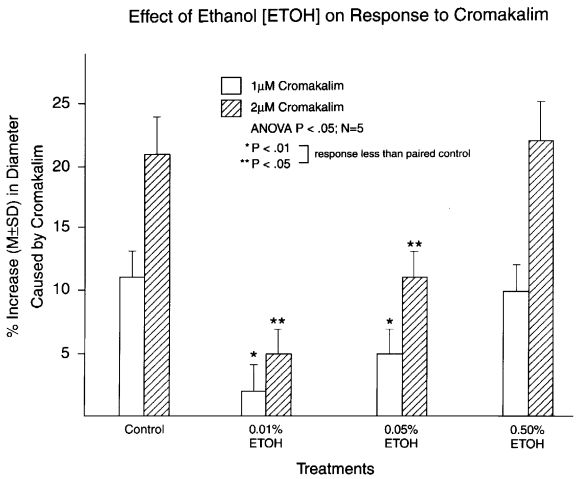

Fig. 4. Ethanol inhibits dilation by cromakalim. $N = 5$ rats. There were 20 arterioles with diameter 40 ± 2 μm (mean ± S.D.).

3.2.4. Time required to produce ethanol effect

To determine how long an incubation period was required to produce an inhibitory effect of ethanol, we tested the response to pinacidil 5 and 10 min after the beginning of incubation with 0.01%. With this dose of ethanol, there was no effect after 5 min, but there was almost total blockade of the response after 10 min. Before ethanol, the response to 1 and 2 μM pinacidil was dilation of 7 ± 1% and 16 ± 1% (mean ± S.D.), respectively. After 5 min of ethanol, the dilations were essentially unaffected (7 ± 2% and 15 ± 2%). After 10 min of ethanol, repeat application of pinacidil produced dilations of only 1 ± 1% and 3 ± 2%, respectively, $P < 0.01$, paired t; $N = 5$.

3.2.5. Ethanol at high dilutions but not lesser dilutions inhibits the response to cromakalim

Fig. 4 shows that ethanol also inhibits dilation by cromakalim. Again, inhibition was absent at the lesser dilutions (higher concentrations) of ethanol. There was no effect of ethanol itself on diameter.

4. Discussion

To our knowledge, these results provide the first demonstration in any species of an inhibitory effect of ethanol on the dilation of cerebral arterioles that would otherwise be elicited by openers of the K_{ATP} ion channel. In addition, the present study provides the first evidence in the rat of an inhibitory effect of dimethylsulfoxide or L-cysteine on the response to an opener of the K_{ATP} ion channel. Inhibition by ethanol and dimethylsulfoxide was gone 15 min after washout (data not shown). The effect of L-cysteine was tested after 30 min of washout and was no longer present (data not shown).

A previous study from this laboratory (Wei et al., 1998) had already demonstrated the effect of dimethylsulfoxide

and L-cysteine in the cat. Two channel openers were used, pinacidil and cromakalim, and both were inhibited. The present study began with a successful attempt to reproduce some of these results in a different species, the rat. After finding that dimethylsulfoxide and L-cysteine did, indeed, inhibit the response to pinacidil, we elected to forego additional attempts at replication and passed, instead, to a study of the effects of ethanol, which is, like dimethylsulfoxide, a commonly used solvent for water-insoluble drugs and, like both dimethylsulfoxide and L-cysteine, an antioxidant.

In the previous study (Wei et al., 1998), three different antioxidants inhibited dilation by the openers of the K_{ATP} channel. The antioxidants were L-cysteine, dimethylsulfoxide and salicylate. In addition, oxidizing agents were found to dilate the vessels (Wei et al., 1996, 1998) and this could be inhibited by glyburide, a known inhibitor of the K_{ATP} channel. From all these data, it was concluded (Wei et al., 1996, 1998) that the K_{ATP} channel could be opened by oxidants acting at an oxidant-sensitive site on the channel.

In the present study we expressed the concentration of dimethylsulfoxide and ethanol as a percentage of the diluting aqueous solution because this is the way in which their contribution to the diluting vehicle is usually expressed in the microvascular literature. When expressed on a molar basis, we find that the effective concentration of dimethylsulfoxide was approximately 5 mM or about 500 times higher than that required in cats (Wei et al., 1998).

The effective doses of dimethylsulfoxide in the present study are within the range known to scavenge hydroxyl radical in vitro (Beckmann et al., 1990; Winterbourn, 1987). Therefore, we cannot rule out the possibility that in this study of rats, the antioxidants were acting against endogenous oxidizing agents whose action may be required to facilitate opening of the K_{ATP} channel by pinacidil. This possibility is at variance with the suggestion made in the earlier study of cats where the effective concentration of dimethylsulfoxide was 1000 times lower than that required to directly scavenge the hydroxyl radical in vitro (Beckman et al., 1990; Winterbourn, 1987); a finding which led (Wei et al., 1998) to the suggestion that the dimethylsulfoxide was not interacting directly with an oxygen-centered reactive species but was, instead, interacting directly with an oxygen-sensitive site on the K_{ATP} channel.

Ethanol also inhibited the response to both pinacidil and cromakalim. As was the case for dimethylsulfoxide, this effect was seen at very low concentrations like those encountered as final concentrations in solutions employing ethanol to solubilize water-insoluble drugs. The inhibitory effect decreased as the dose increased, until no trace of an inhibitory action remained. Selectivity of the inhibitory effect was demonstrated by showing that the dilation produced by sodium nitroprusside was not affected. The earlier study had shown that the effects of dimethylsulfoxide and L-cysteine were likewise selective and not manifested against sodium nitroprusside (Wei et al., 1998).

Since ethanol is an antioxidant, the present findings at low concentrations of ethanol are consistent with the previous conclusion (Wei et al., 1996, 1998) that agents with antioxidant properties will inhibit the dilating action of known openers of the K_{ATP} ion channel. However, if this hypothesis explains the behavior of the affective concentrations of ethanol, it has to be supplemented by a post hoc addition; namely that some additional property, not identified in this study, of ethanol at higher doses, nullifies the effect of its antioxidant characteristics on the K_{ATP} ion channel.

These data and those of the earlier study (Wei et al., 1998) lead to three extremely important suggestions that are relevant to experimental design. The first concerns the use of vehicle controls in studies using water-insoluble agents thought to interact with the K_{ATP} ion channel. The present results with ethanol indicate that performing a vehicle control study at a high concentration of solvent may be an insufficient control when the drug being tested is also employed in dilutions that reduce the concentration of solvent below that of the original control solution. It is necessary to use vehicle controls with exactly the same dilutions of solvent as those employed throughout a study to avoid the false sense of security produced by the absence of an effect of the lowest dilution (i.e. higher concentration) of the solvent.

Second, in determining whether the preparation is significantly altered by the use of ethanol, and presumably dimethylsulfoxide, the duration of exposure to the solvent must be considered in addition to its concentration. In the present study, pinacidil was initially used in final concentrations of ethanol below 0.3%. When we discovered the inhibitory effect of ethanol at these low concentrations, we compared the responses to those with pinacidil dissolved in higher, noninhibitory concentrations of ethanol. We found only a nonsignificant, slight diminution of the response when pinacidil was dissolved in the lower concentration of ethanol. However, in that study, there was no preincubation period. Under those circumstances, the pinacidil solution, containing ethanol, is in contact with the brain for no more than 4 min. A follow-up study preincubated the vessels with a low, potentially inhibitory concentration of ethanol and found that the 5 min of incubation had no effect on the response to pinacidil. Ten minutes of incubation, on the other hand, caused almost complete blockade of the response. Thus, the effect of ethanol depends both on dose and duration of exposure to the ethanol.

The third, and least obvious but equally important, suggestion resulting from our data concerns what they imply for all experiments that employ drugs dissolved in dimethylsulfoxide or in ethanol. There is a potentially large number of responses that are dependent upon the K_{ATP} ion channel. When an investigator is not directly

studying the role of the K_{ATP} channel, that investigator would not ordinarily test the ability of nonaqueous solvents to inhibit the action of channel openers. However, when ethanol or dimethylsulfoxide is used as solvent, the K_{ATP} channel may be inhibited. A preparation is not normal if its K_{ATP} ion channels are unduly resistant to opening. Since K_{ATP} channel-dependent responses have only begun to be identified (Quale et al., 1997; Faraci and Heistad, 1998), it is impossible to eliminate the possibility that results have been and are being altered by an effect of solvents on K_{ATP} channels in cases where ethanol and dimethylsulfoxide have or are being used as solvents. To avoid unintended or unrecognized use of preparations with inhibited channel opening, it is hoped that our new data and our comments will lead investigators who employ either dimethylsulfoxide or ethanol as solvent to first test them against a K_{ATP} channel opener, using the concentration of solvent and the duration of exposure to solvent that will be employed in the experiment to follow. This should be done not only when studying a response known to be dependent upon the channel but in any experiment where the literature is silent about the dependence of the studied response on the channel. Such a control would establish whether, under the conditions of that particular experiment, results will be obtained under circumstances in which the ability to open the K_{ATP} channel is presumably impaired.

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