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Rapid report

Activation by methylene blue of large Ca^{2+}-activated K^+ channels

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Abstract

Using the patch-clamp method, we found that methylene blue (MB), a free radical inhibitor of guanylyl cyclase, activated large, Ca^{2+}-activated K^+ channels (BK_{Ca}) in either cell attached or excised, inside-out patches of human mesangial cells in culture. Since BK_{Ca} are important feedback regulators of contraction of smooth muscle and mesangial cells, these results indicate that MB may be an important opener of BK_{Ca} channels and a regulator of vascular volume and resistance.

Keywords: Methylene blue; Potassium ion channel; Ca^{2+}-activated (BK_{Ca}); Guanylyl cyclase inhibitor; LY83583; Patch clamp

It has been documented in several studies that vasorelaxants, such as nitric oxide (NO) and atrial natriuretic peptide, attenuate contraction of smooth muscle [1–3] and mesangial cells [4,5], in part, by activating BK_{Ca} via a cGMP-dependent signal transduction pathway. In our laboratory, it was shown that the guanylyl cyclase inhibitor, LY83583, inhibited the activation of BK_{Ca} by nitroprusside, an NO donor [4]. However, methylene blue, a free radical inhibitor of guanylyl cyclase, failed to inhibit the activation of BK_{Ca} by nitroprusside [4]. We therefore determined if methylene blue directly activates BK_{Ca} in human mesangial cells. These results could partially explain the controversial finding of direct activation of BK_{Ca} by nitric oxide and the conflicting reports regarding the multiple effects of methylene blue on smooth muscle and mesangial cell tone [6–9]. Moreover, because BK_{Ca} are important regulators of contraction of vascular smooth muscle and glomerular mesangial cells, an opener of these channels would be expected to influence blood pressure and volume and could thereby have clinical and therapeutic significance.

Mesangial cells were isolated from human kidneys and cultured using the following standard techniques [10]. Mesangial cells were plated in Waymouth's culture media (pH = 7.4), supplemented with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 2.0 mM glutamine, 0.66 U/ml insulin, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, antibiotic solution (100 units/ml penicillin and 100 μg/ml streptomycin) and 17% (v/v) newborn calf serum. Subpassages four through ten were used in all experiments. It was shown by Mene et al. [11] that this generation span of MC maintain a constant phenotype and typical smooth muscle-like spindle shape. Moreover, our laboratory has shown that these generations maintain the consistent transcription [12]

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and expression of BK_{Ca} responsive to cGMP-activated protein kinase [5,13].

Mesangial cells were prepared for single channel analysis using standard patch-clamp techniques previously described [13,14]. Pipette and bath solutions contained (in mM): 10 Hepes (pH = 7.4), 2 $MgCl_2$ and either 140 KCl or 135 NaCl plus 5 KCl. The free Ca^{2+} concentration of the bath (initially 1.0 mM) was adjusted to lower concentrations by buffering with EGTA as described previously [15].

After obtaining gigaohm seals, current recordings were made with the electrode on the cell surface (cell attached) or after withdrawing the patched membrane (excised, inside-out). The unitary current (i; defined as zero for C) was determined as the mean of the best fit Gaussian distribution of the amplitude histograms. Channels were considered in an open state (S) when the current was $>(n-1/2)i$ and $<(n+1/2)i$, where n is the number of current levels. The open state probability of a channel (P_o) is defined as the time spent in S divided by the total time of the recording. In all cases, $-V_p$ is the holding potential relative to the pipette.

All chemicals were purchased from Sigma (St. Louis), except LY83583, which was purchased from Calbiochem (La Jolla, CA). For paired data and multiple comparisons, the paired t-test and an analysis of variance plus the Student–Newman–Keuls test, respectively, were used to establish statistical differences ($p \leq 0.05$).

Table 1
The effect of methylene blue (MB) and LY83583 on the activation of BK_{Ca} by nitroprusside

Agents	P_o
Control (13)	0.03 ± 0.01
Nitroprusside (6)	0.38 ± 0.07 *
Nitroprusside + LY83583 (4)	0.03 ± 0.01
Nitroprusside + MB (6)	0.46 ± 0.07 *

Values are open probability of BK_{Ca} in cell attached patches bathed with 135 mM NaCl plus 5 mM KCl and 1 mM $CaCl_2$. $-V_p = 80$ mV. Nitroprusside = 0.1 mM, methylene blue = 0.1 mM, LY83583 = 1.0 mM. * $p \leq 0.05$ vs. control (ANOVA + SNK test).

Fig. 1 shows current tracings of BK_{Ca} in cell attached patches. As shown in Fig. 1A, nitroprusside (0.1 mM) activated BK_{Ca} from a P_o of < 0.05 to a peak of 0.57. Over the next two minutes BK_{Ca} activity returned to baseline. As shown in Fig. 1B, addition of nitroprusside in the presence of methylene blue (0.1 mM) activated BK_{Ca} to a sustained P_o of 0.47. As shown in Fig. 1C, BK_{Ca} were not activated by nitroprusside in the presence of LY83583 (1.0 μM).

A summary of the effects of methylene blue and LY83583 on the activation of BK_{Ca} by nitroprusside is shown in Table 1. In cell attached patches, nitroprusside activated BK_{Ca} significantly in the absence or presence of methylene blue. However, nitroprusside failed to activate BK_{Ca} in the presence of LY83583.

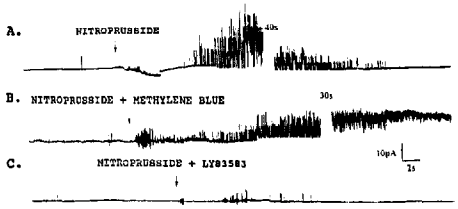

Fig. 1. (A) Current tracings of BK_{Ca} in cell attached patches ($-V_p = 80$ mV) with 135 mM NaCl plus 5 mM KCl in the bath. Addition of nitroprusside (0.1 mM) to the bathing solution ([Ca] = 1.0 mM) caused an activation of BK_{Ca} from a P_o of < 0.05 to a peak of 0.57; after 1 min BK_{Ca} activity returned to baseline. (B) Nitroprusside was added, under the same conditions, except in the presence of methylene blue (0.1 mM). BK_{Ca} were activated to a sustained $P_o = 0.47$. (C) In the presence of LY83583 (1.0 μM), BK_{Ca} were not activated by nitroprusside ($P_o < 0.05$).

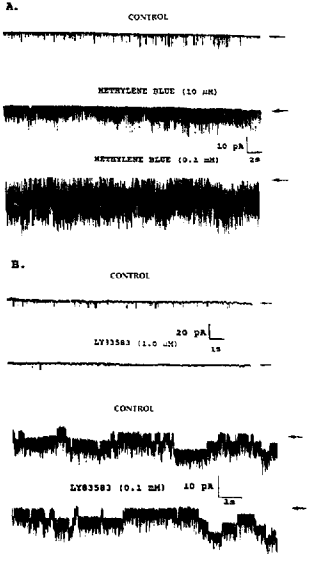

Fig. 2. (A) Tracings of BK_{Ca} in a representative excised, inside-out patch ($-V_c = -40$ mV and $[Ca^{2+}]_i = 1.0$ μM) showing the effects of methylene blue. In the upper tracing, P_o increased from 0.08 to 0.22 upon addition of 10 μM methylene blue. In the lower tracing, 0.1 mM methylene blue activated BK_{Ca} to 0.54. (B) Current tracings showing that LY83583 did not affect BK_{Ca} under the same conditions. Open probability was < 0.05 before and after the addition of LY83583 (top tracing = 1.0 μM, bottom tracing = 0.1 mM).

The representative current tracings (excised, inside-out) of Fig. 2A show that methylene blue activated BK_{Ca} directly in a dose-dependent manner. Upon addition of 10 μM and 0.1 mM methylene blue, P_o increased from 0.08 to 0.22 and 0.54, respectively. In six paired, excised, inside-out experiments ($-V_p = -40$ mV, $[Ca^{2+}]_i = 1.0$ μM) methylene blue significantly ($p < 0.001$) activated BK_{Ca} from a P_o of 0.09 ± 0.02 to 0.45 ± 0.05. In contrast, as shown by the representative current tracings of Fig. 2B, LY83583 (0.1 mM and 1.0 μM) did not affect BK_{Ca}. In four paired experiments, LY83583 did not activate BK_{Ca} ($p = 0.64$). Thus, methylene blue, but not LY83583, activated BK_{Ca} directly from the intracellular side.

It was found that 0.1 mM methylene blue did not affect significantly ($p = 0.18$) solution pH (7.37 ± 0.01 and 7.38 ± 0.01, respectively, before and after addition of methylene blue). Thus, the effects of methylene blue on BK_{Ca} cannot be attributed to changing solution pH.

As an inhibitor of guanylyl cyclase [16,17], methylene blue has been used in several studies to determine the role for cGMP in relaxation of smooth muscle cells [18–20]. BK_{Ca} channels hyperpolarize the membrane and inhibit contraction of mesangial cells [4] and vascular smooth muscle [22] by reducing voltage-gated Ca entry. Although some studies show that methylene blue inhibits arterial relaxation by cGMP-stimulating relaxants [18–21], other studies show methylene blue either had no effect [7–9] or augmented NO-induced relaxation of vascular smooth muscle [6]. It is possible that these conflicting studies are due to simultaneous inhibition of guanylate cyclase and activation of BK_{Ca} channels.

The finding that methylene blue directly activates BK_{Ca} can partially explain the effects reported by Bolotina, who found that NO activated BK_{Ca} after addition of methylene blue [23]. Although Bolotina also found a direct activation by NO of BK_{Ca} in excised patches, it is likely that many intracellular compounds and enzymes, such as guanylyl cyclase and protein kinase G are present in the excised patch. A recent study in this laboratory provided evidence that cGMP-activated kinase was present with BK_{Ca} in excised patches [4]. The absence of direct BK_{Ca} activation by NO was also supported by the recent study by McCobb et al. who found no direct effect of nitroprusside on the gene product for *hslo*, a human BK_{Ca} of vascular smooth muscle expressed in *Xenopus* oocytes [24].

It is postulated that methylene blue inhibits soluble guanylyl cyclase by oxidation of its heme center [20]. However, because methylene blue is an electron acceptor, multiple actions, other than specifically inhibiting guanylyl cyclase, would be expected. Methylene blue causes the release of noradrenaline from adrenergic nerve endings [25], inhibits production of

prostacyclin in arteries [26], and inhibits release of NO from organic nitrates [27].

In conclusion, our results can explain some of the paradoxical and ambiguous effects of methylene blue on the response of vascular cells to relaxing agents. Based on these results, LY83583 would be a more suitable agent for determining the signaling pathways for relaxing agents. The notion that methylene blue can open BK_{Ca} channels could have therapeutic consequences for regulating glomerular filtration rate or vascular smooth muscle tension.

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