A Possibility That the ATP-Sensitive Potassium Channel in Coronary Artery Has a High-Affinity Internal Binding Site for Tetraalkylammonium

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ABSTRACT—The functionally responsible sites for the blocking action of tetraalkylammonium ions (TAAs) in ATP-sensitive K+ (KATP) channels opened by levcromakalim were estimated in canine coronary artery. Tetraethylammonium (TEA) and tetrabutylammonium (TBA) inhibited the levcromakalim-induced relaxation in a noncompetitive manner. Analyses of the noncompetitive antagonism revealed that the binding constant of TBA was about 900 times lower than that of TEA, although the reported affinity of TBA for the internal binding site in various K+ channels was only 10 times higher than that of TEA. TBA is much more lipid-soluble and permeable through membranes than TEA. Thus, TBA blocks KATP channels by binding to a possible high-affinity internal site for TAAs, whereas TEA seems to bind to the external site.

Keywords: ATP-sensitive K+ (KATP) channel, Levcromakalim, Tetraalkylammonium ion

Symmetric tetraalkylammonium ions (TAAs) are frequently used K+ channel blockers. 86Rb+ efflux through ATP-sensitive K+ (KATP) channels stimulated by cromakalim was inhibited by extracellularly applied TAAs in rat aorta, and the inhibitory potency of TAAs increased as the alkyl chain length was increased from methyl to pentyl (1). Thus, lipophilicity of TAAs seems to play an important role in their ability to block KATP channels. Because the permeability through membranes depends mainly on their lipophilicity, it is likely that TAAs act on the internal binding site in the KATP channel. Recently, however, it has been reported that there are two (external and internal) binding sites for TAAs in the pore-forming region of various cloned K+ channels (2-4). The differential blocking potency of TAAs might be due to the different affinities to the two binding sites for TAAs in the KATP channel. We examined the antagonism of two TAAs, tetraethylammonium (TEA) and tetrabutylammonium (TBA), that have different lipophilicity and permeability through membranes against relaxation induced by the specific KATP channel opener levcromakalim (5, 6) and explored the binding sites for TAAs in KATP channels in canine coronary artery.

Circumflex coronary arteries were obtained from 16 mongrel dogs of either sex, weighing 9-12 kg, anesthetized with sodium pentobarbital (30 mg/kg, i.v.). The coronary arteries isolated from the hearts were cut into 3- to 4-mm-long ring segments. The intimal surface of the ring segments was gently rubbed to remove the endothelium. Each ring was suspended in a 25-ml organ bath containing modified Krebs solution of the following composition: 119 mM NaCl, 4.7 mM KCl, 1.17 mM MgSO4, 25 mM NaHCO3, 1.18 mM KH2PO4, 2.5 mM CaCl2 and 11 mM glucose at 37°C, aerated with 95% O2 and 5% CO2. Changes in the force of contraction were measured by force transducers. After a 1.5-hr equilibration with a load of 20 mN, the arterial rings were exposed to TEA or TBA and then contracted by addition of KCl to normal solution to make a final concentration of 25 mM KCl. After the increased force of contraction had reached a plateau about 1.5-2 hr later, levcromakalim was administered in a cumulative manner to construct concentration-relaxation curves. In each experiment, 6 preparations were usually run in parallel.

TEA chloride and TBA chloride (Wako, Osaka) were diluted to the desired concentrations with distilled water. Levromakalim (BRL 38227; SmithKline Beecham, Worthing, UK) was dissolved in 70% ethanol solution to obtain a 10 mM stock solution. On the day of the experiment, this stock solution was diluted to the destined concentrations with distilled water. The highest concentrations of ethanol had no effect on the 25 mM KCl-induced contractions.

Values were expressed in terms of the mean±S.E.M.
The force of contraction produced by KCl was expressed in mN, and the relaxant effects of levocromakalim were expressed as % relaxation (abolition of the KCl-induced force of contraction = 100%). In each preparation, the concentration-relaxation curve for levocromakalim was fitted to a logistic equation:

$$E = \frac{\text{Max} \times A^p}{(A^p + K^p)}$$

where E is the normalized effect, Max is the maximum effect, A is the concentration of levocromakalim, K is the EC50 value and p is the slope parameter. EC50 was presented as pD2 (pD2 = -log EC50). Since TEA and TBA antagonized the relaxation effect of levocromakalim in a non-competitive manner, we employed the analyses of non-competitive antagonism (7). Statistical significance of differences between mean values was estimated by analysis of variance (ANOVA) followed by Dunnett's test or by paired Student's t-test. A P value less than 0.05 was considered to be significant.

TEA (3 -10 mM) or TBA (0.3 – 3 mM) induced a slight contraction in the coronary artery. However, the contraction was much smaller than that induced by 25 mM KCl. In the presence of TEA (3 -10 mM), the KCl-induced contraction was increased (Fig. 1A), whereas TBA (1 – 100 μM) had no effect (Fig. 2A). In the presence of higher concentrations of TBA (0.3 – 3 mM), the force of contraction induced by 25 mM KCl was unstable, i.e., initially it was increased and then gradually declined; thus we examined the effect of TBA on levocromakalim-induced relaxation at the concentrations of 1 – 100 μM. Levocromakalim (0.01 – 30 μM) produced a concentration-dependent relaxation in arteries contracted with 25 mM KCl. The maximum effect of levocromakalim was about 90%. Levocromakalim-induced relaxation was antagonized by TEA or TBA in a non-competitive manner; the maximum effects were suppressed and EC50 values of levocromakalim were increased (Figs. 1A and 2A, Table 1). The effect of TEA (10 mM) on the force of contraction could be com-

![Graph A](image)

**Fig. 1.** Effect of tetraethylammonium (TEA) on levocromakalim-induced relaxation and analyses of non-competitive antagonism. **A:** The noncompetitive antagonism of TEA against the levocromakalim-induced relaxation in canine coronary arteries. Concentration-relaxation curves for levocromakalim in arteries contracted with KCl (25 mM) in the absence (○) and presence of TEA at 0.3 (●), 1 (□), 3 (■) or 10 mM (▲). Data points are the mean ± S.E.M. of 6 experiments for each. Inset: Contractions (means ± S.E.M. in mN) of arteries produced by 25 mM KCl in the absence and presence of TEA. Figures under the columns are the concentrations of TEA in terms of -log M. *P < 0.05, compared with the contraction in the absence of TEA (C: control). **B-D:** Regression lines obtained by the noncompetitive analyses (7). [A] and [A']: the concentrations of levocromakalim in the absence and presence of TEA, respectively. The slopes and intercepts were used to determine the dissociation constant of levocromakalim (Kd) and binding constant of TEA (Kb), which are reported in Table 1.
Fig. 2. Effect of tetrabutylammonium (TBA) on levocromakalim-induced relaxation and analyses of noncompetitive antagonism. A: The noncompetitive antagonism of TBA against the levocromakalim-induced relaxation in canine coronary arteries. Concentration-relaxation curves for levocromakalim in arteries contracted with KCl (25 mM) in the absence (○) and presence of TBA at 1 (●), 3 (□), 10 (■), 30 (△) or 100 μM (▲). Data points are the mean ± S.E.M. of 6 experiments for each. Inset: Contractions (means ± S.E.M. in mN) of arteries produced by KCl in the absence and presence of TBA. Figures under the columns are the concentrations of TBA in terms of - log M. Contractions in the absence of TBA were not significantly different from those in the presence of TBA. B-D: Regression lines obtained by the noncompetitive analyses (7). [A] and [A']: the concentrations of levocromakalim in the absence and presence of TBA, respectively. The slopes and intercepts were used to determine the dissociation constant of levocromakalim (K_A) and binding constant of TBA (K_B), which are reported in Table 1.

Table 1. Effects of tetraethylammonium or tetrabutylammonium on the levocromakalim-induced relaxation in canine coronary artery

| TEA (mM) | Control | 0.3 | 1 | 3 | 10 |
|----------|---------|-----|---|---|----|
| Max      | 88.0±2.7| 85.0±6.8| 67.4±2.8*| 63.9±3.3*| 48.8±6.6*|
| pD_2     | 6.91±0.14| 6.74±0.14| 6.34±0.06**| 6.33±0.09**| 6.22±0.12**|
| pK_A      | —       | 5.65 | 5.97 | 6.04 |
| pK_B      | —       | 3.55 | 3.01 | 2.72 |
| P_B       | —       | 0.78 | 0.77 | 0.84 |

| TBA (μM) | Control | 1 | 3 | 10 | 100 |
|----------|---------|---|---|----|------|
| Max      | 89.0±3.6| 78.1±4.6| 61.7±6.0**| 46.4±7.0**| 28.8±5.3**| 20.4±6.2**|
| pD_2     | 6.83±0.07| 6.80±0.13| 6.43±0.07| 6.09±0.14**| 5.87±0.08**| 5.89±0.17**|
| pK_A      | —       | 6.45 | 6.19 | 6.32 |
| pK_B      | —       | 5.72 | 5.66 | 5.30 |
| P_B       | —       | 0.62 | 0.82 | 0.86 |

Values are expressed in terms of the mean ± S.E.M. (n=6). *P<0.05 and **P<0.01, compared with control values. Estimated by the analyses of noncompetitive antagonism (7): pK_A = -log K_A (levocromakalim), pK_B = -log K_B (TEA or TBA), P_B: fraction of K_A channel blocked by TEA or TBA.
pletely washed out by normal solution (resting force: 0.2±0.3 mN at 10 min after washout), whereas TBA (100 μM) generated a significant increase in resting force (6.0±0.3 mN, P<0.01, at 10 min after washout) at the end of the experiments.

Analyses of noncompetitive antagonism by TEA or TBA against the effect of levocromakalim revealed the binding constant (K_B) of TEA or TBA and the dissociation constant (K_A) of levocromakalim (Figs. 1 and 2, Table 1). The pK_B (=-log K_B) values estimated for TEA (1–10 mM) and TBA (3–30 μM) were 2.72–3.55 and 5.30–5.72, respectively. The pK_A (=-log K_A) values of levocromakalim obtained in the presence of TEA or TBA were 5.65–6.04 or 6.19–6.45, respectively (Table 1). The K_A values of levocromakalim were higher than those of the control EC_{50} values, suggesting the existence of spare receptors or channels for levocromakalim to produce relaxation in canine coronary artery as was the case with the inhibition of contraction by K⁺ channel openers in cardiac muscles (7, 8).

In the present study, levocromakalim-induced relaxation was antagonized by TEA and TBA in a noncompetitive manner in canine coronary artery. The blocking potency (K_B values) of TBA was 500–900 times stronger than that of TEA when the extent of suppression of the maximum effect of levocromakalim was the same (Figs. 1 and 2, Table 1). Various TAAs applied intracellularly, such as TEA and lipid-soluble TBA and tetraptamylanmonium (TPeA), block K⁺ channels at the same site with different affinity. The apparent dissociation constants (K_D) of TEA, TBA and TPeA were 0.36, 0.032 and 0.039 mM, respectively, in squid axon K⁺ current (9); and those of TEA and TPeA were 1.4 mM and about 0.14 mM, respectively, in K_{ATP} channels in skeletal muscle (10). The potency ratio of TEA to TBA or TPeA was around 10. The following question arises: Why does extracellularly applied TBA act much more potently than TEA on the K_{ATP} channels in the coronary artery?

There are two (internal and external) binding sites for TAAs in the pore-forming region of various cloned K⁺ channels (2–4). The external binding sites for TAAs in various K⁺ channels interact usually most potently with TEA but only weakly with TBA (1, 11). Thus, it is unlikely that TBA acts from outside the membrane. TEA seems to act on the external site in the K⁺ channels, since the effect of TEA could be easily washed out. Taglialatela et al. (3) have suggested that lipid-soluble TPeA can diffuse across the membrane and act, with high affinity, on the internal site in the K⁺ channels. Since TBA is as lipid-soluble as TPeA (12), TBA may diffuse across the membrane and may act on the internal site in the K_{ATP} channel. The effects of TBA at high concentrations could not be washed out in canine coronary artery (13) as in this study. Thus, it is highly likely that TBA acts, with high affinity, on the internal site in K_{ATP} channels opened by levocromakalim.

Although the K_B value (2–5 μM) of TBA for the K_{ATP} channels in coronary artery is lower than that in cardiac muscle (pA_2=4.7, K_B=20 μM) (7) and much lower than the K_D value of TPeA (0.14 mM) in skeletal muscle (9), it is almost the same as the IC_{50} value (4 μM) in rat aorta (1). Recently, it is reported that internally applied TEA blocks the Ca^{2+}-dependent K⁺ channels with high sensitivity (14), suggesting a difference in the internal pore (15) of the channels in rat cerebral artery compared with other tissues. Thus, the internal binding site for TAAs in the K_{ATP} channels in vascular smooth muscles seems different from that in cardiac or skeletal muscles.

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