Antagonism by lipophilic quaternary ions of the K⁺ channel opener, levcromakalim, in vascular smooth muscle

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1 The aim of this study was to characterize the interaction between the K⁺ channel opener levcromakalim (LKM) and several quaternary ions, in vascular smooth muscle, *in vitro*. Segments of isolated, thoracic aorta of the rat were suspended in organ baths filled with Krebs solution at 37°C. Cumulative concentration-response curves to LKM were obtained in the absence and in the presence of increasing concentrations of quaternary ions using a number of agents to pre-constrict the vessel. The ions tested were tetraphenylphosphonium (chloride TPP-Cl and bromide TPP-Br salts), tetrapentylammonium (TPeA), tetraethylammonium (TEA), tetraphenylarsonium (TPAs) and tetraphenylboron (TPB).

2 For the compounds which antagonized the vasorelaxation responses of LKM, 'apparent pK_B' values were estimated on the basis of a single concentration of antagonist. These were then used to obtain the following order of potency: TPP-Br (7.22 ± 0.25) = TPAs (7.12 ± 0.04) = TPP-Cl (7.11 ± 0.15) > TPeA (6.23 ± 0.20). TEA and TPB were both found to be inactive at the maximum concentrations used.

3 The interaction between the cationic TPP and anionic TPB was also investigated. The shift in the LKM concentration-response curve constructed in the presence of both of these compounds was compared to that when each agent was present separately. We found that TPB, at concentrations greater than 1 µM, reversed the blockade of the LKM-mediated relaxation induced by TPP (3 µM).

4 Similar experiments were undertaken combining TPB with either alinidine or glibenclamide (both functional antagonists of K⁺ channel openers). It was found that TPB (10 µM) partially reversed the antagonism induced by alinidine (30 and 100 µM) but had no effect on the action of glibenclamide (3 µM).

5 These studies show that lipophilic cations such as TPP and TPAs are potent antagonists of levcromakalim-mediated vasorelaxation responses in the rat thoracic aorta. The mechanism by which these compounds cause their antagonism is not known. However, given the lipophilicity of these compounds, it is possible they may act at a number of sites including the K_{ATP} channel itself or possibly via some other intracellular mechanism.

Keywords: Levcromakalim; smooth muscle relaxation; quaternary ions; K⁺ channels; K⁺ channel antagonists; rat thoracic aorta; tetraphenylphosphonium; tetraphenylboron

Introduction

Levcromakalim is representative of a new class of smooth muscle relaxants (Edwards & Weston, 1993), which act to open a type of K⁺ channel present in the cell membrane resulting in membrane hyperpolarization. These K⁺ channels have been likened to an ATP-sensitive K⁺ (K_{ATP}) channel similar to the ATP-sensitive K⁺ channel first identified in the heart (Noma, 1983). This idea has come about through the observation that potent sulphonylurea-based antagonists of the ATP sensitive K⁺ channel in the pancreatic β-cell, such as glibenclamide (see Ashford, 1990), are also able to antagonize the vasorelaxant response to the K⁺ channel openers in vascular smooth muscle (see McPherson, 1993; Edwards & Weston, 1993). Several years ago we reported that phentolamine (an imidazoline), and alinidine (an imidazolidine) and other structurally related compounds, are also able to antagonize the relaxation induced by the K⁺ channel openers in vascular smooth muscle (McPherson & Angus, 1989; 1990). Subsequent studies have shown that a number of chemically diverse compounds are able to antagonize the functional effects of K⁺ channel openers in vascular smooth muscle and other tissues (see McPherson, 1993; Edwards & Weston, 1993).

Quaternary ions, in particular tetraethylammonium (TEA), have also played an important role in the characterization

and classification of various K⁺ channels (Cook & Quast, 1990), although this particular quaternary ion has a low potency (>1 mM) on the K_{ATP} channels described above. Another area where quaternary ions have been extensively used is in the measurement of membrane potential. It has long been known that some quaternary ions, such as tetraphenylphosphonium (TPP), can partition into cells due to their lipophilic nature. This property has allowed researchers to use [³H]-tetraphenylphosphonium ([³H]-TPP) as a tool for assessing membrane potential in cells because [³H]-TPP partitions between the inside and outside of the cell in proportion to the charge difference (Grinius *et al.*, 1970; Haydon & Hladky, 1972). We recently applied this technique to measure the effect of levcromakalim on membrane potential in cultured smooth muscle cells. It was noted however, in control experiments, that TPP antagonized the functional effects of this compound. Consequently we have performed more detailed studies using a number of selected quaternary ions and found that two lipophilic quaternary ions, TPP and tetraphenylarsonium (TPAs) are potent antagonists of levcromakalim-mediated vasorelaxation responses in the rat thoracic aorta. In addition, we have shown that the blockade of levcromakalim-mediated responses caused by TPP can be reversed by the negatively charged quaternary ion, tetraphenylboron (TPB). The studies show that lipophilic quaternary ions such as TPP and TPAs are some of the most potent antagonists of levcromakalim-mediated actions.

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Methods

Tissue source

Male Wistar-Kyoto rats (WKY), aged between 10–14 weeks, were killed by 20% O₂ and 80% CO₂ narcosis and exsanguination. The thoracic aorta was rapidly removed and placed in ice-cold Krebs solution (composition in mM: NaCl 119, KCl 4.7, MgSO₄·7H₂O 1.17, KH₂PO₄ 1.18, CaCl₂ 2.5, NaHCO₃ 25, and glucose 5.5). The aorta was cleared of connective tissue and cut into 3 mm long segments. Each segment was then suspended on two stainless steel wire hooks, 400 µm in diameter, in 25 ml jacketed glass organ baths filled with Krebs solution aerated with 5% CO₂ in O₂ and maintained at 37°C. The lower hook was fixed to a support leg attached to a micrometer, while the upper wire hook was suspended from a Grass FT03C force transducer through which changes in isometric force were recorded. Force recordings were displayed on a 2-channel flat-bed chart recorder (Model 320 W + W Scientific Instruments, Switzerland). Vessels were left to equilibrate under zero force for 30 min after which an initial force of 5 g was then applied. After another 30 min the force was re-adjusted to 5 g and the tissues were left for a further 30 min. A potassium depolarizing solution (KPSS: composition in mM: KCl 123, MgSO₄·7H₂O 1.17, KH₂PO₄ 1.18, CaCl₂ 2.5, NaHCO₃ 25 and glucose 5.5) was then added to obtain a maximal contractile response of the vessel. This response was used as the standard vasoconstrictor response. After a plateau to KPSS was reached, vessels were washed twice and left until the response returned to the initial baseline, before commencing the experiment.

Each aortic ring was submaximally constricted with a vasoconstrictor (in most studies this was KCl, 25 mM). Once a plateau response to the constrictor was reached, a cumulative concentration-response curve to levcromakalim (LKM) (0.01–30 µM, 0.5 log increments) was then constructed. Concentrations of the relaxant were added when the response to the previous concentration had reached a plateau. In some cases LKM did not cause maximal tissue relaxation, especially if an antagonist was present. Consequently, sodium nitroprusside (SNP; 100 µM) was added at the end of each curve to obtain full tissue relaxation. In studies investigating antagonist action the test compound was added 20 min prior to constricting the tissue with KCl (25 mM; unless specified otherwise). Only one concentration-response curve was constructed on any one aortic ring segment.

Analysis of results

The contraction to the submaximal constrictor (e.g. KCl) was taken as 100% response and relaxation produced by different concentrations of the vasodilator (i.e. LKM or SNP) was expressed as a percentage of this response. The percentages obtained were then represented graphically and the pD₂ (–log EC₅₀) value calculated as the concentration of dilator required to cause 50% relaxation of the maximal relaxant response. In the cases where the maximal response had not been obtained (i.e. in the presence of high concentrations of the antagonists) the response to the highest concentration of the dilator was taken as the maximal response. To determine whether the antagonists directly affected the tissue's ability to contract, the response to the constrictor was expressed as a percentage of the maximal KPSS contraction.

It was observed that at high concentrations, most of the active quaternary ions shifted the LKM concentration-response curve in a non-parallel fashion. Also, the slope of the curve was reduced and the maximum response to LKM appeared to be depressed. This suggested that the type of antagonism displayed by the quaternary ions was non-competitive, preventing us from the use of Schild analysis (Jenkinson, 1991) as a means of determining the potency of

the active ions. Instead, an 'apparent pK_B' was estimated, based on a single concentration of antagonist.

The following equation was used:

$$\text{'Apparent pK}_B\text{'} = -\log\left(\frac{[\text{antagonist Conc., M}]}{[\text{concentration ratio} - 1]}\right) \quad (1)$$

The single concentration of the antagonist used to calculate the 'apparent pK_B' was selected on the basis that it produced a 'parallel' shift in the LKM concentration-response curve without overtly affecting the maximal LKM response.

One-way repeated measures analysis of variance (ANOVA) was used for comparison of several dependent groups and one-way ANOVA was used to test several values which were independent. Either Student-Newman-Keuls test (for all pairwise multiple comparisons) or Dunnett's method (for comparisons for all groups vs control) were used as post-ANOVA tests. All statistical calculations were performed using SigmaStat (Jandal Scientific, U.S.A.). Results in the text are given as mean ± s.e.mean.

Figure 1 Chemical structures of the quaternary ions and the K⁺ channel opener, levcromakalim. They are (from top to bottom) levcromakalim, tetraphenylphosphonium, tetraphenylarsonium, tetraethylammonium, tetrapentylammonium and tetraphenylboron.

Drugs

Drugs used and their sources were: levcromakalim (Beecham, UK); sodium nitroprusside (David Bull, Australia); methoxamine hydrochloride (Sigma, U.S.A.); 11 α , 9 α -epoxymethano-prostaglandin H₂ (U46619, The Upjohn Co., Kalamazoo, U.S.A.); glibenclamide (Hoechst, Australia); alinidine hydrobromide (Boehringer-Ingelheim, Germany); tetraethylammonium chloride hydrate, tetrapentylammonium iodide, tetraphenylphosphonium chloride, tetraphenylphosphonium bromide (Aldrich, USA); tetraphenylarsonium chloride, tetraphenylboron sodium (Sigma, U.S.A.).

Results

Antagonism of levcromakalim responses by quaternary ions

Six quaternary ions were tested for the ability to antagonize LKM-mediated vasorelaxation. The structures of these compounds are shown in Figure 1. The potency of each active ion was estimated by calculating the 'apparent pK_B ' value. The results from these calculations are summarized in Table 1. Four of the compounds: tetraphenylphosphonium bromide (TPP-Br), tetraphenylphosphonium chloride (TPP-Cl), tetraphenylarsonium (TPAs) and tetrapentylammonium (TPeA), were able to block LKM-induced vasorelaxation (Figure 2). The two tetraphenylphosphonium salts, TPP-Br and TPP-Cl (Figure 2a), at concentrations as low as 1 μ M, produced a significant shift in the LKM concentration-response curve. Statistically significant shifts in the LKM curve were also associated with 1 and 3 μ M TPAs (Figure 2b and Table 1).

Tetrapentylammonium (TPeA) produced a significant shift in the LKM concentration-response curve at a concentration of 10 μ M (Figure 2c); however, this response was markedly less than that produced by the two TPP salts and TPAs (see Table 1). It should be noted that TPeA, at 3 μ M and greater, acted directly on the smooth muscle to constrict it. This was reflected by a change in tone generated to KCl (25 mM), which increased from approximately 30–40% to 60% of the KPSS contraction. In contrast, none of the other quaternary ions tested directly affected the basal tone or that induced by KCl (25 mM) which remained constant at between 30–40%.

The negatively charged tetraphenylboron (TPB), at concentrations ranging from 0.1 to 10 μ M, was found to be inactive as was tetraethylammonium (TEA) at concentrations up to 100 μ M (Table 1).

Characterization of tetraphenylphosphonium chloride (TPP-Cl)-induced antagonism

Since TPP-Cl was found to be quite a potent antagonist of the vascular actions of LKM, we performed a number of

additional studies with this compound to characterize the type of antagonism displayed by the quaternary ions.

Time to equilibrium and reversibility studies In separate studies, the optimal incubation time for the quaternary ions was determined. TPP-Cl (3 μ M) was selected as the represen-

Figure 2 Mean levcromakalim (LKM) concentration-response curves obtained in the rat isolated thoracic aorta in the absence (○) and in the presence of increasing concentrations of the quaternary ions. (a) Tetraphenylphosphonium chloride; (b) tetraphenylarsonium; (c) tetrapentylammonium. The concentrations used were 0.1 (●), 0.3 (▽), 1 (▼), 3 (□) and 10 (■) μ M. Error bars are $\pm$ s.e.mean, and if not visible are contained within the symbol ($n = 4$).

Table 1 Summary of the pD_2 and 'apparent' pK_B values obtained for the quaternary ions tested for levcromakalim (LKM) antagonistic activity, in rat isolated thoracic aorta constricted with KCl (25 mM) or methoxamine (●) (3 μ M)

Compound	Control $pD_2^{(1)}$	Antagonist (μ M) ⁽²⁾	+ Antagonist $pD_2^{(3)}$	'Apparent' $pK_B^{(4)}$
TPP-Br	7.34 $\pm$ 0.14	1	6.08 $\pm$ 0.30	7.22 $\pm$ 0.25
TPP-Cl	7.55 $\pm$ 0.07	1	6.40 $\pm$ 0.13	7.11 $\pm$ 0.15
●TPP-Cl	7.10 $\pm$ 0.08	3	5.57 $\pm$ 0.22	7.03 $\pm$ 0.12
TPAs	7.34 $\pm$ 0.07	1	6.19 $\pm$ 0.09	7.12 $\pm$ 0.04
TEA	7.50 $\pm$ 0.12	100	7.43 $\pm$ 0.03	NA
TPeA	7.53 $\pm$ 0.12	3	6.72 $\pm$ 0.15	6.23 $\pm$ 0.20*
TPB	7.63 $\pm$ 0.02	10	7.54 $\pm$ 0.04	NA

⁽¹⁾ pD_2 value to LKM obtained in the absence of antagonist.

⁽²⁾Concentration of the quaternary ion used to calculate 'apparent pK_B '.

⁽³⁾ pD_2 values obtained in presence of the antagonist, at the concentration given in the previous column.

⁽⁴⁾'Apparent' pK_B values estimated for each of the active compounds, calculations based on the single concentration of antagonist given.

*Mean significantly different from other means ($P < 0.05$, one way ANOVA and Student-Newman-Keuls test). $n = 4$.

NA: not active.

tative quaternary ion in this series of experiments and was left in the organ bath for a period of 10, 20, 30, 45 and 60 min prior to constricting the vessel with KCl (25 mM). The concentration-effect curves to LKM, obtained in the presence of TPP-Cl (3 μ M), were superimposable at all times of pre-incubation studied, indicating that equilibrium antagonism was achieved rapidly (<10 min). A 20 min pre-incubation was chosen in subsequent experiments including those described above.

We were also interested in testing the reversibility of the TPP-Cl antagonism. TPP-Cl (3 μ M) was incubated for 20 min and then the vessels were repeatedly washed (at intervals of 5 min) for a period of 30, 60 and 120 min, before the tissues were preconstricted and LKM concentration-response curves constructed. It was found that even after extensive washing for a period up to 120 min, TPP-Cl (3 μ M) antagonism was still evident (Figure 3).

Vasoconstrictor and vasodilator dependency Methoxamine (3 μ M) was also used to constrict the aorta to determine whether the antagonism produced by TPP-Cl was dependent on the constrictor agent used. TPP-Cl, at concentrations as low as 1–3 μ M, produced a significant shift in the LKM concentration-response curve in these vessels (Figure 4) in a manner similar to that observed in KCl constricted tissues (Table 1). As in the previous study, the pretreatment of the tissue with TPP did not alter the ability of methoxamine to constrict the tissue which was approximately 60–70% of the maximum response to KPSS in all cases.

We also wanted to determine whether TPP-Cl inhibited only LKM responses or whether it affected the actions of other vasodilators as well. The same protocol as described above was followed, except that sodium nitroprusside (SNP) was used to obtain stepwise tissue relaxation responses. TPP-Cl at concentrations ranging from 0.1 μ M to 10 μ M, had no significant effect on the action of SNP on KCl (25 mM) or methoxamine (3 μ M) preconstricted tissues ($n = 4$).

Effect of the negatively charged tetraphenylboron (TPB) on selective levcromakalim antagonists

Effect of TPB on TPP-induced levcromakalim antagonism While performing experiments with TPP-Cl, we noticed that the negatively charged quaternary ion in our group,

Figure 3 Reversibility of tetraphenylphosphonium chloride (TPP-Cl) antagonism: mean levcromakalim (LKM) concentration-response curves in the absence (open symbols) and in the presence (closed symbols) of TPP-Cl (3 μ M) incubated for 20 min followed by washing at 5 min intervals for 30 (●), 60 (▼) and 120 (■) min ($n = 4$). Control curves constructed in the absence of TPP where treated identically to those obtained in the presence of TPP in terms of washing. Error bars are $\pm$ s.e.mean and if not visible are contained within the symbol.

TPB, was able to reverse the antagonism produced by TPP-Cl. A typical experiment showing the interaction between the two ions is shown in Figure 5. In KCl (25 mM)-constricted tissues, TPB at concentrations greater than 1 μ M, reversed the antagonism caused by TPP-Cl (3 μ M). At concentrations of 10 μ M and 100 μ M, TPB produced a full reversal of TPP-Cl action (Figure 6). In another series of experiments, we constricted the vessels with methoxamine (3 μ M) and again found that TPB (10 μ M) was able to reverse fully the blockade of LKM action produced by TPP-Cl (3 μ M).

Figure 4 Mean levcromakalim (LKM) concentration-response curves obtained in the absence (○) and in the presence of increasing concentrations of tetraphenylphosphonium chloride (TPP-Cl; 3 (●) and 10 (▼) μ M) in the rat thoracic aorta preconstricted with methoxamine (3 μ M) ($n = 4$). Error bars are $\pm$ s.e.mean, and if not visible are contained within the symbol.

Figure 5 Interaction between tetraphenylboron (TPB) and tetraphenylphosphonium chloride (TPP-Cl) in the rat isolated thoracic aorta. (a) A representative trace showing the vasorelaxation induced by increasing concentrations of levcromakalim (LKM, 0.01–30 μ M) in rat thoracic aorta constricted with KCl (25 mM) in the absence of any antagonist. (b) Same as (a) except tetraphenylphosphonium chloride (TPP-Cl) (3 μ M, 20 min, incub.) was present. (c) Same as (b) except that tetraphenylboron (TPB) (10 μ M) was added in addition to the TPP-Cl (3 μ M) (both drugs added 20 min prior to KCl constriction). At the end of each LKM concentration-response curve, sodium nitroprusside (SNP, 100 μ M) was added to obtain full tissue relaxation. W = wash.

Figure 6 Interaction between tetraphenylphosphonium chloride (TPP) and tetraphenylboron (TPB). Mean levcromakalim (LKM) concentration-response curves obtained in the absence (○) and in the presence (●) of TPP (3 μ M) and increasing concentrations of TPB, in the rat isolated thoracic aorta precontracted with KCl (25 mM). TPB concentrations used = 0 (○), 0.1 (∇), 1 ($\blacktriangledown$), 10 ($\square$) and 100 ($\blacksquare$) μ M, $n = 4$. Error bars are $\pm$ s.e.mean, and if not visible are contained within the symbol.

Figure 7 Interaction between tetraphenylboron (TPB) and other levcromakalim (LKM) antagonists, glibenclamide and alinidine. Mean LKM concentration-response curves obtained in KCl (25 mM) constricted rat isolated thoracic aorta in the absence and in the presence of (a) Glibenclamide, control LKM (○); plus glibenclamide (3 μ M, ∇); plus TPB (10 μ M, ●); glibenclamide and TPB (10 μ M, $\blacktriangledown$); $n = 5$. (b) Alinidine, control LKM (○); plus TPB (10 μ M; ●); plus alinidine (30, ∇ and 100, $\square$ μ M); alinidine (30, $\blacktriangledown$ and 100, $\blacksquare$ μ M) plus TPB (10 μ M); $n = 4$. Error bars are $\pm$ s.e.mean, and if not visible are contained within the symbol.

Effect of TPB on glibenclamide and alinidine mediated levcromakalim antagonism Following the observation that TPB could reverse TPP-Cl antagonism of LKM responses, we investigated whether the same type of reversal occurred after combining TPB with other antagonists of levcromakalim in particular glibenclamide (sulphonylurea) and alinidine (imidazolidine).

Glibenclamide (3 μ M), caused a rightward shift in the LKM concentration-response curve, which was not reversed when the antagonist was combined with TPB (10 μ M) (Figure 7a). In the absence of any antagonist the pD_2 for LKM was 7.15 ± 0.17 , with glibenclamide (3 μ M) pD_2 was 5.38 ± 0.09 and with both glibenclamide and TPB the pD_2 was 5.53 ± 0.2 ($n = 4$). The interaction between alinidine and TPB was studied at two concentrations of alinidine (Figure 7b). Alinidine (30 μ M) produced a significant shift in the LKM curve which was partially reversed in the presence of TPB (10 μ M). In the absence of any agent LKM had a pD_2 of 7.08 ± 0.09 . In the presence of alinidine (30 μ M) the pD_2 was 5.57 ± 0.14 . When alinidine and TPB were combined together the pD_2 was 6.01 ± 0.23 ($n = 4$) which was significantly more potent (by a factor of 2.7) than in the presence of alinidine alone ($P < 0.05$). A similar phenomenon was also noted when using alinidine (100 μ M) (control $pD_2 = 7.08 \pm 0.09$; plus alinidine $pD_2 = 5.27 \pm 0.16$; alinidine plus TPB $pD_2 = 5.56 \pm 0.20$; $n = 4$; $P < 0.05$).

Discussion

The major finding from this study is that some but not all quaternary ions possess the ability to antagonize vasorelaxation responses mediated by levcromakalim in rat isolated thoracic aorta. In addition we have also found that the negatively charged quaternary ion, TPB, is able to interfere with the antagonistic activity of TPP and to a lesser extent, alinidine, on LKM-mediated responses.

The antagonism displayed by the quaternary ions

The nature of the antagonism displayed by the quaternary ions is similar to that displayed by the imidazol(id)ines (Challinor & McPherson, 1993). We have recently shown that imidazol(id)ine based compounds, such as alinidine, are antagonists of K^+ channel openers such as cromakalim, in both vascular and non-vascular smooth muscle (McPherson & Angus, 1989; 1990; Challinor & McPherson, 1993). The antagonism displayed by imidazol(id)ine compounds is characterized by a shift to the right in the cromakalim concentration-effect curve associated with a flattening of the curve at higher concentrations ($\geq 10 \mu$ M) and a reduction in the maximum response (McPherson & Angus, 1989; 1990; see also Murray *et al.*, 1989; Good *et al.*, 1992). This type of antagonism was also displayed by the active quaternary ions. This contrasts with the results obtained with sulphonylurea compounds such as glibenclamide which cause a parallel shift in the curve with no effect on the maximum response (see Challinor & McPherson, 1993). Other studies showed that the time of onset of the antagonism displayed by TPP was rapid (< 10 min) and was slowly reversible.

The number of compounds tested did not allow extensive structure-activity relationships to be performed. There is no doubt however, that there is a strong correlation between the structure of the ions and their potency as LKM antagonists. Each of the three active quaternary ions: TPP (Br and Cl salts), TPAs and TPcA – shifted the LKM concentration-response curve in what appeared to be a non-parallel fashion. Because of this we did not perform Schild analysis. Instead we calculated an 'apparent pK_B ' value to obtain an estimate of compounds' potency. It must be stressed that these values serve only as an approximate indicator of potency and they do not represent the pK_B values normally obtained for true competitive antagonists. By calculating their 'apparent' pK_B

values, we found that the positively charged quaternary ions, containing the phosphonium or arsonium ion at the centre and phenyl residues as side chains, (i.e. TPP-Cl, TPP-Br and TPAs, see Figure 1), were more potent antagonists of LKM action than the ammonium based compounds. This could be due to a combination of factors. For example both phosphonium and arsonium ions can readily delocalize their positive charge onto the phenyl groups, making the whole quaternary molecule very lipophilic, which in turn may allow the ion to interact effectively with the potassium channel (see later). In addition, the mass of the quaternary ion seems to play an important role in determining its potency. Since both TPP and TPAs contain phenyl rings, it is likely that these side chains are important for high potency. This theory is further supported by the observation that molecules such as tetrapentylammonium (TPeA), which carry less compact five carbon pentyl side chains, or tetraethylammonium (TEA) which has only two carbon ethyl groups, displayed little potency (TPeA) or indeed were found to be inactive (TEA).

Quast & Webster (1989) have investigated the effect of several ammonium based quaternary ions on cromakalim-induced $^{86}\text{Rb}^+$ efflux in the rat aorta. They also found TEA to be an active blocker of cromakalim albeit at concentrations of approximately 1.0 mM, higher than those used in our experiments. The same researchers also investigated the relationship between quaternary ion structure of ammonium based compounds and their ability to antagonize cromakalim-induced $^{86}\text{Rb}^+$ efflux (see also Cook & Quast, 1990). Their findings showed that the potency of these compounds increased with increasing chain length, up to the n-pentyl compound, however the potency was reduced if the chain length was increased further. They suggested that the interaction site of the channel has a strong preference for bulky, but not too bulky, lipophilic cations. In their studies they found the n-pentyl compound had an IC_{50} for inhibiting cromakalim-induced $^{86}\text{Rb}^+$ efflux of approximately 0.1 μM . This value indicates a potency for TPeA which is approximately 5–10 times greater than that calculated for the same compound blocking LKM-mediated vasorelaxant responses in this study. At the present time we do not have any explanation for this discrepancy; however, it has been noted that some, but not all, antagonists block cromakalim-mediated $^{86}\text{Rb}^+$ efflux at concentrations much lower than those needed to block functional effects in the same tissue (Quast, 1993). In addition, the fact that TPeA was able to activate the smooth muscle directly indicates that the actions of this particular ion are complex and may thus account for some of the discrepancies in the results. However, the potency of TPeA was much less, by a factor of 10 fold, than that displayed by TPP and TPAs.

Mechanism of action of the quaternary ions and their lipophilic properties

It is thought that K^+ channel openers, such as levcromakalim, exert their effect by opening a type of K_{ATP} channel in a variety of tissues (see McPherson, 1993; Edwards & Weston, 1993). This mechanism of action of these compounds has been established primarily through the finding that sulphonylureas, such as glibenclamide, a potent and selective K_{ATP} channel blocker (see Ashford, 1990) in the pancreas, are able to antagonize the actions of the K^+ channel openers in vascular and non-vascular smooth muscle (McPherson & Angus, 1990; see Cook & Quast, 1990; Longman & Hamilton, 1992). The most obvious mechanism of action is that the quaternary ions block K_{ATP} channels to prevent K^+ ion efflux from the cell. What we know at present about the interaction between quaternary ions and the potassium channels, is largely based on studies performed with tetraethylammonium (TEA). Despite its relatively low potency, TEA has been the most commonly used non-selective potassium channel blocker (see Cook & Quast,

1990). The exact mode of interaction between the quaternary compounds and the K_{ATP} channel is still unknown. It has been proposed that the action of TEA, on other types of K^+ channels, is to interact directly with the ion channel to plug it and prevent ion movement, i.e. the so called 'plug' hypothesis (Heginbotham & McKinnon, 1992). While it is possible that the quaternary ions simply act as ion channel plugs of K_{ATP} channels, these compounds are known to have complex actions in the cell and the possibility exists that they exert their effects through another, or perhaps a combination, of mechanisms.

As mentioned previously, some of the quaternary ions, such as tetraphenylphosphonium (TPP) and tetraphenylarsonium (TPAs), are highly lipophilic. This allows them to enter the hydrophobic core of the membrane and delocalize their charge in order to allow passive equilibration with the electrical potential across the membrane. Because of this property, these lipophilic cations have been used in the past to measure the polarity of membrane potentials in biological systems (Grinius *et al.*, 1970; Haydon & Hladky, 1972). Studies examining the mode of action of TPP in this respect have found it to have complex intracellular effects which raises the possibility that these quaternary ions may not solely act as ion channel plugs to antagonize the effects of levcromakalim. For example TPP is known to concentrate in mitochondria (see Saito *et al.*, 1992). It has been also shown to interfere with Na,K-ATPase activity (Klodos & Plesner, 1992). More detailed electrophysiological studies are obviously required to deduce the precise mechanism of action of the quaternary ions such as TPP and TPAs.

Whatever the mechanism of action of TPP and TPAs they represent two of the most potent antagonists of the functional actions of K^+ channel openers identified so far, having a potency similar to that of glibenclamide (see Challinor & McPherson, 1993).

Mechanism of action of a negatively charged tetraphenylboron (TPB)

Although TPB was shown to be inactive as an LKM antagonist, it did reverse the antagonism produced by other quaternary ions such as TPP-Cl and also partially reversed the antagonism of alinidine. The simplest explanation for this phenomenon is that the compounds are displaying chemical antagonism, a situation where the two drugs react chemically to form a product that cannot interact with the receptor (Kenakin, 1987). Thus the negatively charged TPB forms a non-active complex with TPP or alinidine (Timmermans *et al.*, 1980), both of which are positively charged at physiological pH. This would reduce the amount of free antagonist concentration present in the bath and subsequently would diminish the effect of the antagonist. Since the reversal of the antagonism to alinidine was partial, it is possible that alinidine/TPB complex has lesser affinity than the TPP/TPB complex. Although TPB was shown to reverse the antagonism induced both by TPP and alinidine, it had no effect on the actions of glibenclamide. Glibenclamide, and other sulphonylureas, are negatively charged at physiological pH (Zunkler *et al.*, 1989; Findlay, 1992) and consequently would not interact with TPB which is also negatively charged.

Again the situation is likely to be more complicated. In studies using ^3H -TPP to monitor membrane potential it is common practice to include unlabelled TPB. The inclusion of TPB enhances the equilibration of ^3H -TPP between the inside and outside of the cell. This is thought to result from the uptake of TPB into the lipid membrane which has a preference for negatively charged ions such as TPB (Haydon & Hladky, 1972). The uptake of TPB results in a more favourable environment for positively charged ions, such as TPP, to cross. The presence of TPB within the lipid bilayer may compete with TPP for sites present in the K^+ channel, but since TPB cannot occupy the negatively charged environment of the ion channel itself, it does not impede ion move-

ment (see Sunner *et al.*, 1981; Stauffer & Dougherty, 1988; Dougherty & Stauffer, 1990 for additional details). It is an intriguing possibility that TPB may prove to be an 'un-blocker' of all K⁺ channels (and possibly other ion channels) where the blockade results from channel plugging as may be the case with TEA and TPP. We are currently testing this hypothesis.

In conclusion, we have found that a number of lipophilic quaternary ions antagonize LKM-induced vasorelaxation responses in vascular smooth muscle. The mechanism by which these compounds cause their antagonism is not known. However given the lipophilicity of these compounds it is possible they may act at a number of sites including the K_{ATP} channel

itself or possibly via some intracellular mechanism. We have also demonstrated that tetraphenylboron (TPB), a negatively charged quaternary ion, was able to reverse the effect of the antagonism displayed by TPP and, to a lesser extent, the imidazolidine alinidine. The use of these agents alone and in combination should prove valuable tools for the further study of K⁺ channel openers and the processes they regulate.

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