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Ca^{2+}-dependent K^+ channels are targets for bradykinin B_1 receptor ligands and for lipopolysaccharide in the rat aorta

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Abstract

Although rat aorta smooth muscle cells in culture constitutively express bradykinin B_1 receptors, the normotensive rat aorta does not respond to the bradykinin B_1 receptor agonist des-Arg^9-bradykinin, whereas vessels from the spontaneously hypertensive rat (SHR) respond to bradykinin B_1 receptor agonists with cell membrane hyperpolarization and relaxation. Bacterial lipopolysaccharide also is inactive on the normotensive rat but hyperpolarizes the SHR aorta. To determine whether this could be due to the increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the SHR, we raised $[Ca^{2+}]_i$ in normotensive rats by treatment with thapsigargin. In the thapsigargin-treated aorta, both lipopolysaccharide and des-Arg^9-bradykinin induced hyperpolarization, which was reversed by the Ca^{2+}-dependent K^+ channel inhibitor iberiotoxin and by the bradykinin B_1 receptor antagonists Lys-[Leu^8]-des-Arg^9-bradykinin and [Leu^8]-des-Arg^9-bradykinin. Thus the bradykinin B_1 receptor, as well as lipopolysaccharide, needs activated Ca^{2+}-dependent K^+ channels for functional expression. The two bradykinin B_1 receptor inhibitors, however, have effects on Ca^{2+}-dependent K^+ channels which are not mediated by bradykinin B_1 receptors.

Keywords: Bradykinin B_1 receptor; Lipopolysaccharide; (Rat) aorta; Calcium-dependent K^+ channel

1. Introduction

Bradykinin B_1 receptors are not normally functional, becoming evident only after some types of tissue injury, such as the injection of bacterial materials or under pathological conditions such as inflammation and sepsis (Regoli and Barabe, 1980; McLean et al., 2000). Bradykinin B_1 receptor upregulation is due to de novo protein synthesis, in response to specific cytokines released in situations of trauma and stress (Passos et al., 2004; Marceau and Bachvarov, 1998). Agents that stimulate the synthesis of these cytokines, such as bacterial lipopolysaccharide, also lead to functional bradykinin B_1 receptor expression when administered either in vitro or in vivo (for a recent review see Leeb-Lunderberg et al., 2005).

In the case of the rat aorta, although smooth muscle cells in culture were shown to constitutively express functional bradykinin B_1 receptors (Schaeffer et al., 2001), isolated aorta preparations do not respond to bradykinin B_1 receptor agonists nor to lipopolysaccharide.

Lipopolysaccharide is known to activate large conductance Ca^{2+}-dependent K^+ (K_{Ca}) channels of vascular smooth muscle cell membranes, mediated by inducible nitric oxide synthase (iNOS), leading to hyporeactivity to vasoconstrictor agents (Thiemermann, 1997; Hecker et al., 1999; Muller et al., 2000). However, lipopolysaccharide can also activate K_{Ca} channels in the vascular smooth muscle independently of iNOS induction (Chen et al., 1999, 2000; Yakubovich et al., 2001).

We have previously shown that lipopolysaccharide (Farias et al., 2002), as well as the bradykinin B_1 receptor agonist des-Arg^9-bradykinin (Farias et al., 2004), induce dose-dependent hyperpolarization and relaxation in the aorta of the spontaneously hypertensive rat (SHR), but not in the normotensive Wistar rat. It was suggested that this difference may be due to the increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) present in the SHR (Jelicks and Gupta, 1990), since K_{Ca} channels are constitutively open in this strain (Liu et al., 1997). We have now explored this hypothesis by investigating whether increasing $[Ca^{2+}]_i$ would induce, in the normotensive rat aorta, a

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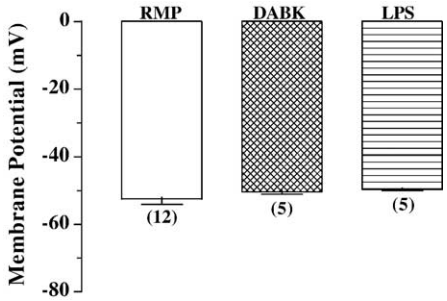

Fig. 1. Membrane potential measured in rat aortic rings. The resting membrane potential (RMP) and the effects of 1 μM des-Arg^9-bradykinin and 10 μg/ml lipopolysaccharide (LPS) are shown. For each aortic ring obtained from individual rats (the number of which is shown in parentheses below the bars), 5 to 10 cells were impaled, and the averages of the respective measurements were used to obtain the means±S.E.M.

behavior similar to that observed in the SHR vessels towards des-Arg^9-bradykinin and lipopolysaccharide. For this purpose we treated the rat aorta with thapsigargin, a selective inhibitor of the sarcoplasmic Ca^{2+}-ATPase (Thastrup et al., 1990) that promotes increase in $[Ca^{2+}]_i$ by emptying the intracellular Ca^{2+} stores. Iberiotoxin, an inhibitor of K_{Ca} channels, was used to demonstrate the role of these channels in the aortic smooth muscle responses, Lys-[Leu^8]-des-Arg^9-bradykinin and [Leu^8]-des-Arg^9-bradykinin were used as bradykinin B_1 receptor antagonists.

2. Materials and methods

2.1. Animals

Experiments were carried out using male normotensive Wistar rats from the Wistar Institute, Philadelphia, PA, USA, inbred at Escola Paulista de Medicina/Federal University of São Paulo, Brazil. The rats were 20–30 weeks old and weighed 250–350 g. They were killed by decapitation, their thoracic aortas were removed, cleaned of adherent connective tissue and cut into rings (3–4 mm length) for electrophysiological measurements. Care was taken to ensure that the endothelial layer was not damaged during tissue preparation. All procedures complied with the norms of the Ethics Committee for Research of the São Paulo Hospital of the Federal University of São Paulo.

2.2. Membrane potential

To observe the relaxant responses of the rat aorta to agonists, the preparations must be pre-contracted with vasoconstrictor agonists which inhibit K^+ channels (Quayle et al., 1997), blunting the relaxant response due the activation of these channels. To avoid this problem, intracellular microelectrodes were used to obtain direct measurement of the smooth muscle cell membrane potential in aortic rings (Nelson et al., 1990). These measurements yield very reproducible values with small deviations.

Micropipettes (borosilicate glass capillaries 1B120F-6, World Precision Instruments, WPI) were made by means of a horizontal puller (Model PN-3, Narishige, Tokio, Japan) and filled with 2 M KCl (tip resistance 20–40 MΩ and tip potential <6 mV). The microelectrodes were mounted in Ag/AgCl half-cells on a micromanipulator (Leitz, Leica) and connected to an electrometer (Intra 767, WPI).

The aortic rings were placed in a 2-ml perfusion chamber containing Krebs–bicarbonate solution of the following composition (in mM): NaCl 122, KCl 5.9, $MgCl_2$ 1.25, $NaHCO_3$ 15, $C_6H_{12}O_6$ 11, $CaCl_2$ 1.25 (pH 7.4). They were superfused at a rate of 3 ml min^{-1} with Krebs solution at 37 °C, aerated with the gas mixture 5% CO_2–95% O_2.

After an equilibration period of 2 h under an optimal resting tension of 1.0 g, the impalements of the smooth muscle cells were made from the adventitial side. The electrical signals were continuously monitored on an oscilloscope (Model 54645A, Hewlett Packard) and recorded in a potentiometric chart recorder (Model 2210, LKB-Produkter AB). The successful implantation of the electrode was evidenced by a sharp drop in voltage upon entry into a cell, a stable potential (±3 mV) for at least 1 min after impalement, a sharp return to zero upon exit, and minimal change (<10%) in microelectrode resistance after impalement.

Membrane potentials were measured before and after stimulation of the vessels with thapsigargin (1 μM), lipopolysaccharide (10 μg/ml) or des-Arg^9-bradykinin (1 μM), in the presence or absence of iberiotoxin (10 nM), Lys-[Leu^8]-des-Arg^9-bradykinin (10 μM) or [Leu^8]-des-Arg^9-bradykinin (10

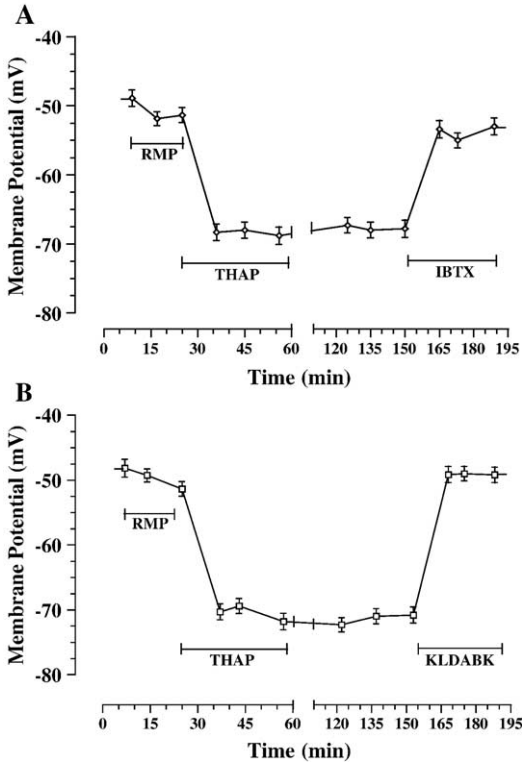

Fig. 2. Typical experiments in which, after recording the resting membrane potential (RMP), the rat aorta was superfused with 1 μM thapsigargin (THAP), causing a hyperpolarization that was not reversed by washing with Krebs solution. Superfusion with 10 nM iberiotoxin (IBTX) (A) or with 10 μM Lys-[Leu^8]-des-Arg^9-bradykinin (KLDABK) (B) caused depolarization to RMP levels.

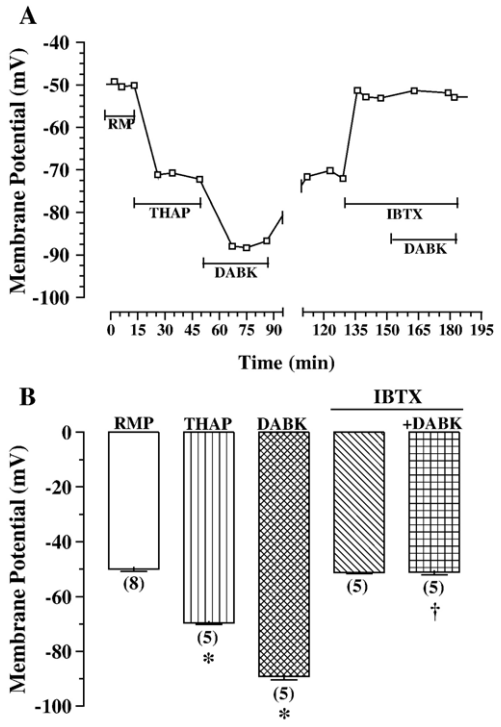

Fig. 3. (A) Representative experiment showing that, after hyperpolarization by previous treatment with 1 μM thapsigargin (THAP), superfusion with 1 μM des-Arg^9-bradykinin (DABK) caused further hyperpolarization that was reversed by washing the preparation. Addition of 10 nM iberiotoxin (IBTX) depolarized the aorta to resting membrane potential (RMP) levels, and further addition of 1 μM des-Arg^9-bradykinin (DABK) to the superfusion medium had no effect on the membrane potential. (B) Bar graph showing the average membrane potential values measured in five experiments similar to that shown in (A). For each aortic ring obtained from individual rats 5 to 12 cells were impaled, and the averages of the respective measurements were used to obtain the means ± S.E.M. *P<0.05 versus respective RMP (Newman–Keuls test). †P<0.05 versus the response to DABK alone (Newman–Keuls test).

μM). The time of contact of the drugs with the preparations before the impalements was 10 min.

The presence of a functional endothelium was tested in all preparations by checking the acetylcholine (10 μM) response which is characteristic of vessels with an intact endothelium (Furchgott, 1981).

2.3. Drugs

Thapsigargin, lipopolysaccharide (*Escherichia coli* lipopolysaccharide, 0111:B4), iberiotoxin and acetylcholine chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Des-Arg^9-bradykinin, Lys-[Leu^8]-des-Arg^9-braykinin and [Leu^8]-des-Arg^9-bradykinin were purchased from Bachem Bioscience Inc. (King of Prussia, PA, USA). The inorganic salts were products of the highest analytical grade from Merck (Darmstadt, Germany).

2.4. Statistical analysis

All data are expressed as means ± S.E.M. with the number of animals in parenthesis. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test in the case of pairwise comparisons between groups. When the data consisted of repeated observations at successive time points, ANOVA for repeated measurements was applied to determine differences between groups. When more than one impalement was made on the same aortic ring from the same rat, the measurements were averaged and considered as n = 1. Differences were considered significant when P<0.05.

3. Results

Although the presence of constitutive bradykinin B_1 receptors has been demonstrated in rat aortic smooth muscle cells (Schaeffer et al., 2001), Fig. 1 shows that the bradykinin B_1 agonist des-Arg^9-bradykinin did not affect the membrane potential of the smooth muscle cells measured in the isolated rat aorta. The figure also shows that the aortic rings did not respond to lipopolysaccharide.

Addition of thapsigargin caused a marked hyperpolarization which was not reversed by washing the

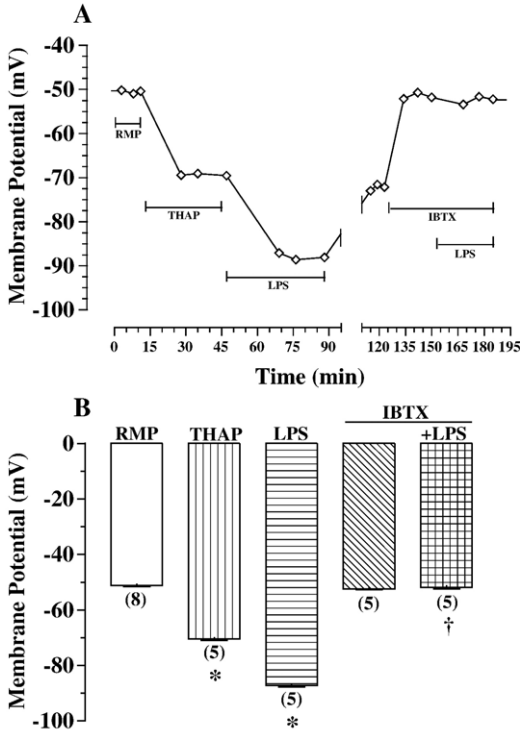

Fig. 4. (A) Representative experiment showing that, after hyperpolarization by previous treatment with 1 μM thapsigargin (THAP), superfusion with 10 μg/ml lipopolysaccharide (LPS) caused further hyperpolarization that was reversed by washing the preparation. Addition of 10 nM iberiotoxin (IBTX) depolarized the aorta to resting membrane potential (RMP) levels, and further addition of 10 μg/ml lipopolysaccharide (LPS) to the superfusion medium had no effect on the membrane potential. (B) Bar graph showing the average membrane potential values measured in five experiments similar to that shown in (A). For each aortic ring obtained from individual rats, 5 to 12 cells were impaled, and the averages of the respective measurements were used to obtain the means ± S.E.M. *P<0.05 versus respective RMP (Newman–Keuls test). †P<0.05 versus the response to LPS alone (Newman–Keuls test).

preparation for at least 2 h (Fig. 2A). This hyperpolarizing effect was completely inhibited by the addition of iberiotoxin, indicating that it was due to stimulation of K_{Ca} channels. Lys-[Leu^8]-des-Arg^9-bradykinin also reversed the hyperpolarization induced by thapsigargin (Fig. 2B), confirming previous evidence that this bradykinin B_1 receptor antagonist has a receptor-independent inhibitory effect on K_{Ca} channels (Farias et al., 2004).

Figs. 3 and 4 show that, during the hyperpolarized state due to previous treatment with thapsigargin, des-Arg^9-bradykinin and lipopolysaccharide elicited further hyperpolarizations that were reversible upon washout. Addition of iberiotoxin restored the normal resting membrane potential and inhibited the hyperpolarization induced by des-Arg^9-bradykinin (Fig. 3) or by lipopolysaccharide (Fig. 4) under the effect of thapsigargin.

We also investigated the effect of Lys-[Leu^8]-des-Arg^9-bradykinin and [Leu^8]-des-Arg^9-bradykinin, inhibitors of the bradykinin B_1 receptor, on the hyperpolarizing responses induced by des-Arg^9-bradykinin and by lipopolysaccharide on the rat aorta. Lys-[Leu^8]-des-Arg^9-bradykinin also restored the normal resting membrane potential and inhibited the hyperpolarization induced by des-Arg^9-bradykinin (Fig. 5A) or by lipopolysaccharide (Fig. 5B) under the effect of thapsigargin. Similar results were observed with [Leu^8]-des-Arg^9-bradykinin (not shown).

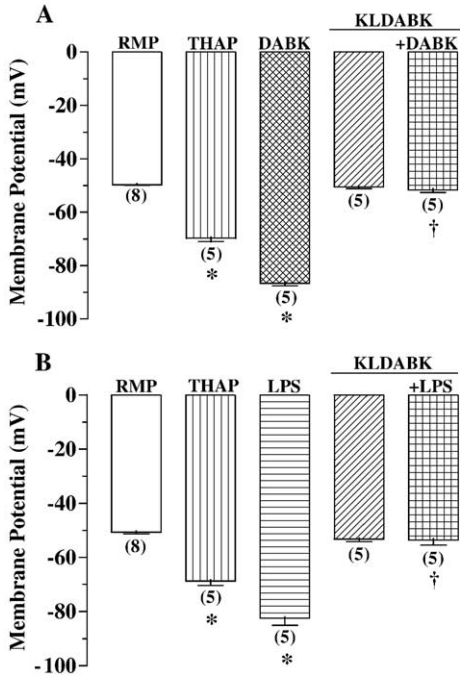

Fig. 5. Membrane potentials measured in rat aortic rings. (A) Effects of 1 μM thapsigargin (THAP), 1 μM des-Arg^9-bradykinin (DABK) and 10 μM Lys-[Leu^8]-des-Arg^9-bradykinin (KLDABK) alone or in the presence of DABK. (B) Effects of 1 μM thapsigargin (THAP), 10 μg/ml lipopolysaccharide (LPS) and 10 μM KLDABK alone or in the presence of LPS. For each aortic ring obtained from individual rats (the number of which is shown in parentheses below the bars), 5 to 10 cells were impaled, and the averages of the respective measurements were used to obtain the means±S.E.M. *$P<0.05$ versus respective RMP (Newman–Keuls test). †$P<0.05$ versus the response to DABK (A) or to LPS (B) (Newman–Keuls test).

4. Discussion

In view of our previous finding that both des-Arg^9-bradykinin and lipopolysaccharide induced hyperpolarization in the SHR, but not in the normotensive rat aorta, we have explored the hypothesis that this difference could be due to the $[Ca^{2+}]_i$ being elevated in the SHR relative to normotensive controls. This increased calcium concentration would activate K_{Ca} channels leading to the hyperpolarized state observed in the SHR aorta. Accordingly, we investigated the effect of increasing the $[Ca^{2+}]_i$ on the membrane potential of the normotensive rat aorta, by treatment with thapsigargin, an inhibitor of the sarcoplasmic Ca^{2+} ATPase.

Thapsigargin hyperpolarized the aortic smooth muscle cell membrane (Fig. 2) and we also found that this effect was not reverted by washing the preparation for at least 2 h. Thus the inhibition of sarcoplasmic Ca^{2+} ATPase, and consequent increase in $[Ca^{2+}]_i$, remained after washout of thapsigargin allowing mimicking, in the normotensive rat aorta, the behavior observed in the SHR vessels (Farias et al., 2004). Indeed, the hyperpolarization measured in thapsigargin-treated normotensive rat aortas was similar to that previously observed in the untreated SHR and in both cases it was inhibited by iberiotoxin, indicating that this effect was due to activation of K_{Ca} channels. The hyperpolarization induced by thapsigargin was also inhibited by Lys-[Leu^8]-des-Arg^9-bradykinin and [Leu^8]-des-Arg^9-bradykinin, indicating that these antagonists of the bradykinin B_1 receptor inhibit the K_{Ca} channels without involving the receptor.

This receptor-independent effect of Lys-[Leu^8]-des-Arg^9-bradykinin on K_{Ca} channels is in agreement with the previous finding that it blocks the responses to lipopolysaccharide in the de-endothelized rat aorta, in which bradykinin B_1 receptors are absent (Farias et al., 2004).

The present results with thapsigargin-treated rat aortas indicate that Lys-[Leu^8]-des-Arg^9-bradykinin and [Leu^8]-des-Arg^9-bradykinin block the responses to both des-Arg^9-bradykinin and lipopolysaccharide by acting on K_{Ca} channels. Therefore, Lys-[Leu^8]-des-Arg^9-bradykinin and [Leu^8]-des-Arg^9-bradykinin not only inhibit the interaction of des-Arg^9-bradykinin with its specific receptor, but also its receptor-independent effects on K_{Ca} channels. For the two agonists to induce their effect, these channels must be either constitutively open (as in the SHR) or previously activated (as in the thapsigargin-treated normotensive rats). This effect, therefore, appears to be due to an increase of the open probability of these channels, as described for the effect of lipopolysaccharide in cerebral arteries (Hoang and Mathers, 1998).

Our results show that des-Arg^9-bradykinin and lipopolysaccharide, which do not normally affect the membrane potential of normotensive rat aortas, induce hyperpolarization after treatment with thapsigargin, indicating that activated K_{Ca} channels mediate that effect. In the SHR aorta, which is hyperpolarized due to constitutive activation of the K_{Ca} channels, the responses to des-Arg^9-bradykinin and lipopolysaccharide are probably due to these agents increasing the mean open time of those channels.

In conclusion, we have shown that the bradykinin B_1 receptor, which is constitutively expressed in the rat aortic smooth muscle cell (Schaeffer et al., 2001), can be functionally expressed when the K_{Ca} channels are activated by increasing the intracellular Ca^{2+} concentration. Both the bradykinin B_1 agonist and lipopolysaccharide induce hyperpolarization by increasing the mean open time of the K_{Ca} channels. This hyperpolarization is probably responsible for the hypotension resulting from the decreased systemic vascular resistance observed in endotoxic shock. Our results also indicate that Lys-[Leu^8]-des-Arg^9-bradykinin and [Leu^8]-des-Arg^9-bradykinin have a direct effect on K_{Ca} channels that is not mediated by bradykinin B_1 receptors.

In view of the role of kinins in inflammation and sepsis, bradykinin B_1 and B_2 receptor antagonists have been developed as possible therapeutic agents against these affections (for a recent review see Leeb-Lunderberg et al., 2005). However, we found that Lys-[Leu^8]-des-Arg^9-bradykinin and [Leu^8]-des-Arg^9-bradykinin inhibit K_{Ca} (this paper) as well as K_{ATP} channels (Farias et al., 2004), blocking the hyperpolarizing effect of lipopolysaccharide or bradykinin B_1 agonists on these channels, independently of interaction with the bradykinin B_1 receptor. This suggests that, in the search for drugs against endotoxic shock, besides the modeling based on the bradykinin B_1 receptor, Lys-[Leu^8]-des-Arg^9-bradykinin and [Leu^8]-des-Arg^9-bradykinin may be good lead compounds for the development of peptide or non-peptide therapeutic agents directed towards the inhibition of the interaction of lipopolysaccharide and kinins with K_{Ca} and K_{ATP} channels.

Acknowledgments

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