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Effect of Diltiazem and Pinacidil on the Response of the Rabbit Urinary Bladder to Repetitive Stimulation and In Vitro Ischemia

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The effect of repetitive stimulation, in the presence and absence of diltiazem or pinacidil, on the contractile responses of isolated strips of rabbit bladder detrusor to field stimulation and carbachol, after 2 hr of incubation in a medium that serves as an in vitro model of ischemia (oxygen and substrate depleted Tyrode's solution), was determined. Our results are summarized as follows: a) The magnitude of the contractile dysfunctions after in vitro ischemia was enhanced by repetitive stimulation. b) Pre-incubation of isolated strips of detrusor with diltiazem (50 μM) inhibited the contractile responses to field stimulation (FS) and carbachol by 43 and 50%, respectively. Pinacidil (100 μM) inhibited the contractile responses to FS and carbachol by 37 and 32%, respectively. c) Neither diltiazem nor pinacidil protected the bladder strips against the effects of 2 hr of incubation in in vitro ischemia medium. However, d) both pinacidil and diltiazem reduced the level of contractile dysfunctions induced by repetitive stimulation. In conclusion, the contractile response to FS was significantly more sensitive to in vitro ischemia and repetitive stimulation than was the contractile response to carbachol. Both diltiazem and pinacidil protected the contractile responses to FS and carbachol from the degenerative effects of repetitive stimulation, but not from the effects of in vitro ischemia. *Neurourol. Urodynam. 18:129–137, 1999.*

Key words: hypoxia; ischemia; hyperreflexia; reperfusion; bladder; free radicals; lipid peroxidation; hyperreflexia; calcium

INTRODUCTION

The urinary bladder is a smooth muscle organ whose function is to collect and store urine at low intravesical pressures and then to periodically expel the urine via a

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highly coordinated sustained contraction [Wein, 1991; Steers, 1992; Andersson, 1993]. The integrity and function of detrusor smooth muscle are extremely sensitive to hypoxia, overdistension, diabetes, and ischemia [Eika et al., 1993; Irwin and Gallowan, 1993; Monson et al., 1994; Uvelius and Arner, 1995; Ohmura et al., 1996, Greenland et al., 1997]. One common factor in these pathologies may be a reduction in blood flow. In this regard, hypertrophy secondary to partial outlet obstruction results in cyclical ischemia (hypoxia)/reperfusion (reoxygenation) associated with micturition [Lin et al., 1993; Siroky et al., 1993; Pontari et al., 1993; Greenland and Brading, 1996; Greenland et al., 1997].

In a recent presentation, Dr. Greenland [1997] demonstrated that in the obstructed pig model, hyperreflexia during bladder filling was associated with brief periods of reduced blood flow and tissue hypoxia. In other biological systems, ischemia followed by reperfusion stimulated the production of free radicals and resulted in increased lipid peroxidation [Kaneko et al., 1994; Dixon et al., 1990].

In vitro studies demonstrated that bladder contractile responses to various forms of stimulation (field stimulated release of neurotransmitters, direct stimulation of muscarinic receptors, and direct membrane depolarization) are progressively inhibited by glucose (substrate) deprivation, hypoxia, and a model of ischemia (hypoxia + substrate deprivation) [Bilgen et al., 1992; Kwon et al., 1996; Pessina et al., 1997]. The response to nerve-mediated stimulation (field stimulation [FS]) is the most sensitive to in vitro glucose deprivation, hypoxia, and a model of ischemia [Kwon et al., 1996; Pessina et al., 1997]. In vitro ischemia was shown to be more damaging to the contractile response to all forms of stimulation than hypoxia alone, and hypoxia alone is more damaging to the contractile response to all forms of stimulation than substrate deprivation [Pessina et al., 1997].

In recent studies, we determined that the contractile response to FS was significantly more sensitive to both in vitro hypoxia and in vitro ischemia than was the contractile responses of either carbachol or KCl. In addition, repetitive stimulation increased the rate of contractile failure under all incubation conditions tested (oxygen + substrate, hypoxia + substrate, and hypoxia − substrate) [Ohnishi et al., 1998]. In a subsequent study, we demonstrated that the level of dysfunction was increased in the presence of high extracellular calcium concentration ($[Ca^{2+}]_{ex}$), and was decreased in the presence of low $[Ca^{2+}]_{ex}$ [Levin et al., in press a, b]. These studies indicate that increased Ca^{2+} may play a part in ischemia-induced contractile dysfunction in vivo. The current studies investigate whether reducing the level of Ca^{2+} entering the cell by blocking Ca^{2+} entry through L-type Ca^{2+} channels or by hyperpolarization with K^{+} channel openers can protect against the contractile dysfunction induced by an in vitro model of ischemia in the presence or absence of repetitive stimulation.

MATERIALS AND METHODS

Tissue Preparation and Equilibration in Normal Oxygenated Tyrode's Solution

Each male New Zealand White rabbit, weighing 3–4 kg, was sedated with ketamine/xylazine (25 mg ketamine, 10 mg xylazine/kg, i.m.); surgical anesthesia was induced with intravenous pentobarbital (25 mg/kg, i.v.). The urinary bladder was rapidly removed through a lower midline incision; the bladder body was separated

from the base at the level of the ureteral orifices. Eight longitudinal muscle strips were cut from the bladder body. Each strip was mounted in a separate 15 ml bath containing Tyrode's solution (NaCl 124.9 m*M*, KCl 12.5 m*M*, $NaHCO_3$ 23.8 m*M*, $MgCl_2 \cdot 6H_2O$ 0.5 m*M*, $NaH_2PO_4 \cdot H_2O$ 0.4 m*M*, and $CaCl_2$ 1.8 m*M*) with glucose (1 mg/ml), equilibrated with 95% O_2, 5% CO_2, and maintained at 37°C. At the end of 30 min of incubation, each strip was stimulated for 15 sec with FS (32 Hz, 80 V, and 1 msec), followed by carbachol (100 μ*M*). The strips were washed three times with fresh Tyrode's solution. Then, strips 1–4 were incubated in the presence of diltiazem (5×10^{-5} *M*) for 30 min, and strips 5–8 were incubated in the absence of diltiazem. At the end of this incubation, all strips were stimulated for 15 sec with FS and then with carbachol. All strips were washed three times with fresh Tyrode's. Diltiazem was added, again, to strips 1–4.

Strips 1, 2, 5, and 6 were subjected to 2 hr of in vitro ischemia by changing the incubation medium to Tyrode's solution without glucose and changing the aeration to 95% N_2, and 5% CO_2. Strips 3, 4, 7, and 8 were maintained in oxygenated medium containing glucose. During the 2-hr in vitro ischemia incubation, strips 1, 5, 3, and 7 were stimulated repetitively for 15 sec with FS (32 Hz) at 5-min intervals. At the end of the in vitro ischemia incubation, all strips were washed three times with fresh, oxygenated buffer containing glucose. Diltiazem was added to strips 1–4, and all strips were incubated for an additional hour in fresh, oxygenated buffer containing glucose, without repetitive stimulation. At the end of the period of reoxygenation plus substrate replacement, each strip was stimulated with FS and carbachol.

A similar sequence of studies was performed in the presence and absence of pinacidil (1×10^{-5} *M*).

Statistics

Statistical significance was determined using analysis of variance with the Neuman-Keuls test for significance between individual groups. A $P < 0.05$ was required for significance.

RESULTS

The effect of pretreating the tissues with diltiazem or pinacidil is presented in Fig. 1. The concentrations of diltiazem and pinacidil chosen for these experiments were those, determined by preliminary studies, that inhibited rabbit bladder contractile responses to FS and carbachol by ~50%. Diltiazem had no effect on the response of the bladder strips to 2 hr of in vitro ischemia (Fig. 2). However, diltiazem significantly reduced the level of contractile dysfunctions induced by repetitive stimulation (Fig. 2). Similarly, pinacidil had no effect on the response of the bladder strips to 2 hr of in vitro ischemia (Fig. 3); pinacidil significantly reduced the level of contractile dysfunctions induced by repetitive stimulation (Fig. 3).

DISCUSSION

Partial outlet obstruction, an animal model for BPH, induces an increase in bladder mass, progressive contractile dysfunctions, progressive denervation, and mitochondrial and sarcoplasmic reticular (SR) damage [Levin et al., 1993, 1995]. Cur-

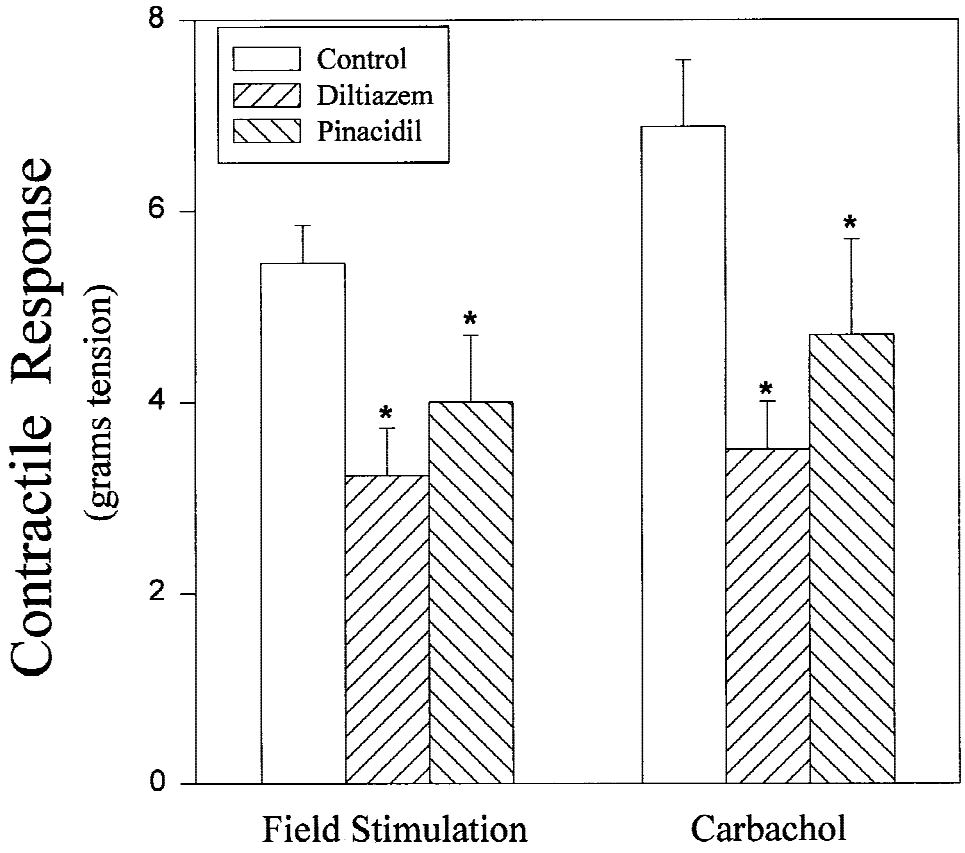

Fig. 1. Effect of diltiazem (5×10^{-5} *M*) and pinacidil (1×10^{-5} *M*) on the contractile response to field stimulation and carbachol. Each column is the mean ± SEM for four to six individual preparations. *, significantly different from control (oxygen); $P < 0.05$.

rent studies demonstrated that the obstruction-induced increased wall thickness results in a cyclical ischemia/reperfusion [Greenland and Brading, 1996, 1997]. In animal models of partial outlet obstruction, a certain percentage of animal bladders develop spontaneous contractile activity, and the rate of contractile failure is generally more rapid in obstructed animals that exhibit spontaneous contractile activity than in animals that are not hyperreflexic [Jorgensen et al., 1993; Mostwin, et al., 1991; Steers et al., 1996; Brading, 1997].

Partial outlet obstruction also induces marked changes in bladder metabolism. There is an increase in anaerobic metabolism, primarily via an increase in lactate dehydrogenase, and a shift in lactate dehydrogenase isoforms, and a decrease in aerobic metabolism. There is a decrease in crossbridge turnover rate, and an increase in efficiency, thus allowing the bladder smooth muscle to work more efficiently in the presence of reduced pO_2 [Uvelius and Arner, 1995, 1997]. In a recent study, DiSanto et al. [1998] demonstrated that partial outlet obstruction in rabbits induced a myosin isoform shift to an isoform that has a low actin-activated ATPase activity. This shift would also contribute to the ability of the obstructed bladder to generate tension in the presence of low oxygen.

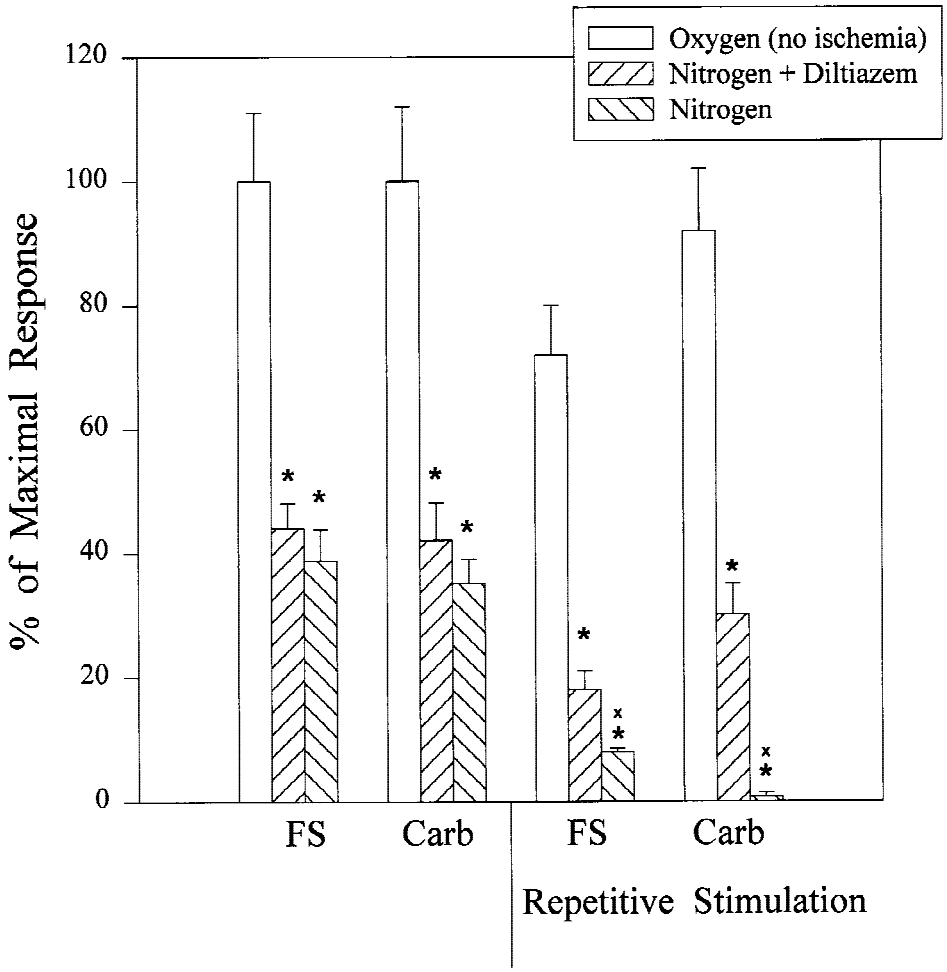

Fig. 2. Effect of 2 hr of in vitro ischemia ± repetitive stimulation followed by 1 hr of re-oxygenation and substrate replacement on the response to diltiazem; 100% (maximal response) equals the contractile response after the 30-min pre-incubation in the presence of diltiazem. The column marked oxygen refers to the contractile response of the strips following incubation for 2 hr in non-ischemic (oxygen + glucose) medium. Each column is the mean ± SEM of between four and six individual experiments. *, significantly different from control (oxygen); x, significantly different from nitrogen + diltiazem; $P < 0.05$.

In vitro studies demonstrated that the rate of development of contractile dysfunction in isolated bladder strips is significantly greater in the presence of an in vitro model of ischemia (hypoxia + glucose deprivation) than in the presence of hypoxia, and the rate of development of contractile dysfunction is significantly greater in the presence of hypoxia than in the presence of glucose deprivation [Pessina et al., 1997]. This correlates with studies that demonstrate that the rate at which ATP decreases with time in glucose-deficient medium is lower than in hypoxic medium, which, in turn, is lower than in in vitro ischemic medium, and that contractile stimulation results in an increased rate of ATP utilization under all conditions [Levin, 1991; Kwon et al., 1996].

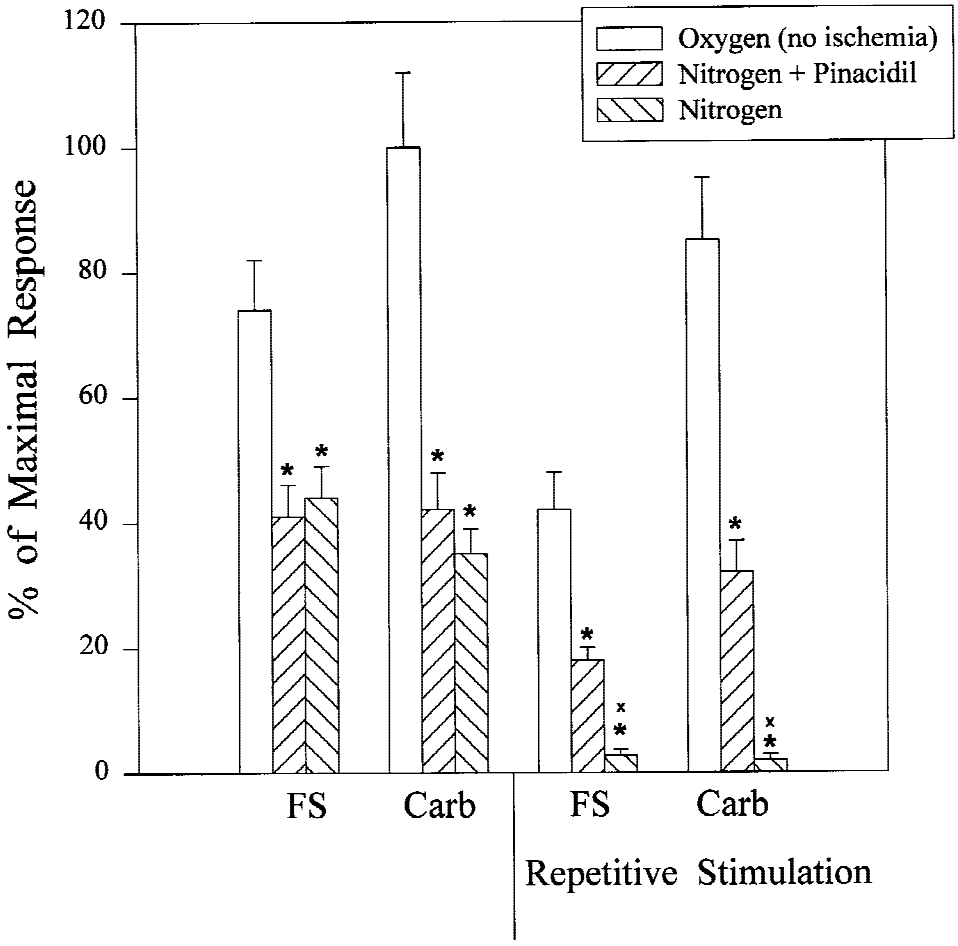

Fig. 3. Effect of 2 hr of in vitro ischemia ± repetitive stimulation followed by 1 hr of re-oxygenation and substrate replacement on the response to pinacidil; 100% (maximal response) equals the contractile response after the 30-min pre-incubation in the presence of diltiazem. The column marked oxygen refers to the contractile response of the strips after incubation for 2 hr in non-ischemic (oxygen + glucose) medium. Each column is the mean ± SEM of between four and six individual experiments. *, significantly different from control (oxygen); x, significantly different from nitrogen + pinacidil; $P < 0.05$.

Ca^{2+} translocation and intracellular Ca^{2+} ($Ca^{2+}{}_i$) release play an important role in mediating the contractile response to autonomic stimulation [Maggi et al., 1988; Mostwin, 1985; Andersson, 1993]. A major participant in the etiology for bladder dysfunction secondary to partial outlet obstruction is believed to be progressive $Ca^{2+}{}_i$ dysregulation and ischemia-induced Ca^{2+} overload. During the periods of cyclical ischemia, free $[Ca^{2+}]_i$ increases, due to both the movement of $Ca^{2+}{}_{ex}$ into the smooth muscle cells, and the release of Ca^{2+} stored in the SR. The increased $[Ca^{2+}]_i$ activates Ca^{2+}-activated hydrolytic enzymes, such as calpain and phospholipase A_2. The resultant activated enzymes disrupt the integrity of specific neuronal, cellular, and subcellular membranes, resulting in the observed progressive denervation, mitochondrial damage, and SR damage [Levin et al., 1993, 1995]. This Ca^{2+}-induced mem-

brane damage results in the progressive reductions in the contractile responses to all forms of stimulation that are characteristic of bladder dysfunction secondary to partial outlet obstruction. Furthermore, hyperreflexia increases $Ca^{2+}{}_i$ cycling, which would be expected to exaggerate Ca^{2+} overload during periods of ischemia and increase the observed progression of bladder dysfunction after partial outlet obstruction. There is excellent evidence that both partial outlet obstruction, and in vivo ischemia result in the activation of calpain, which is consistent with the above hypothesis [Zhao et al., 1997].

Three ways of reducing the stimulated increase in Ca^{2+} translocation into the smooth muscle cells during stimulation are administration of a Ca^{2+} channel blocker, such as diltiazem [Steers, 1992; Andersson, 1993], which inhibits movement of Ca^{2+} through L-type Ca^{2+} channels during smooth muscle stimulation; administration of a potassium channel opener, such as pinacidil, which increases the threshold stimulation required to depolarize the membrane; and administration of a Ca^{2+} chelator, such as EGTA, which reduces the free $[Ca^{2+}]_{ex}$, thus reducing the ability to stimulate a translocation of Ca^{2+} from extracellular to intracellular compartments.

In studies just completed, increasing the $[Ca^{2+}]_{ex}$ significantly increased the rate of contractile failure in the presence of in vitro ischemia, and low $[Ca^{2+}]_{ex}$ or EGTA had significant protective effects. This protective effect was most prominent in the presence of repetitive stimulation [Levin, in press a]. Further studies demonstrated that whereas diltiazem and pinacidil could protect the contractile function from repetitive stimulation during in vitro ischemia, incubation with thapsigargin + ryanodine could not [Levin et al., in press b]. The current study was designed to determine whether diltiazem or pinacidil could protect the bladder from the development of contractile dysfunctions in the absence of repetitive stimulation.

Both diltiazem and pinacidil protected the detrusor strip from the increased rate of contractile failure induced by repetitive stimulation but not from the effect of the in vitro ischemia itself. This indicates that the movements of Ca^{2+} stimulated by repetitive stimulation (calcium cycling) is directly involved in the increased rate of contractile failure observed, and both diltiazem and pinacidil act by inhibition of the calcium cycling.

Consistent with the hypothesis that Ca^{2+} dysregulation plays a major role in the bladder dysfunctions induced by both ischemia and partial outlet obstruction is the study by Steers et al. [1994] showing that treatment of rats with verapamil prior to partial outlet obstruction significantly protected the rats against the development of bladder dysfunction.

In conclusion, bladder contractile dysfunction induced by in vitro ischemia followed by reoxygenation is mediated in part by Ca^{2+} dysregulation. Repetitive stimulation increases the rate and magnitude of contractile dysfunction, primarily by increasing Ca^{2+} cycling within the smooth muscle cells. Pinacidil and diltiazem protected against the increased rate of contractile failure induced by repetitive stimulation but not against the contractile dysfunction induced by in vitro ischemia alone.

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