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The anticholinergic drug propiverine inhibits the protein kinase C activity in the rat urinary bladder

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Received February 26, 2004, accepted April 22, 2004

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Pharmazie 60: 49–51 (2005)

There is ample evidence that non-cholinergic protein kinase C (PKC) mediated signal transduction pathways are involved into regulation of bladder smooth muscle contractions. To evaluate whether the anticholinergic and calcium modulating drug propiverine exerts intracellular effects by inhibition of the PKC, male inbred LEW 1A rats were pretreated with 0.6, 2, 6 and 60 mg/kg body weight for 5 days. Furthermore, competition assays with partially purified PKC were performed with propiverine *in vitro*. The activities of the membrane-bound and soluble PKC were assessed by ^{32}P enrichment of lysine-rich histone. *Results:* The active, membrane-bound PKC decreased by about 60% accompanied by increase of the soluble form after propiverine in doses above 0.6 mg/kg. 100 nM of the drug inhibited the PKC also *in vitro* whereas the propiverine metabolites M5 and M6 and atropine were without any effect. *Conclusions*: Propiverine was identified to be an inhibitor of the protein kinase C. Its contribution to the non-cholinergic control of hyperactive detrusor smooth muscle cells needs further investigation.

1. Introduction

Propiverine [2,2-diphenyl-2(1-propoxy) acetic acid (1-methylpiperid-4-yl) ester] is on the market for the treatment of the neurogenic and idiopathic detrusor hyperactivity which is a major reason of incontinence in many patients with overactive bladder (Andersson et al. 2001). The bladder spasmolytic effect of the drug results from blockade of acetylcholine receptors and influence on calcium homeostasis in detrusor smooth muscle cells. Propiverine is partially selective on muscarinic M3 relative to M2 receptors, antagonizes atropine-resistant, but nifedipine-sensitive detrusor smooth muscle contractions by blockade of voltage dependent Ca^{2+}-channels and inhibits the activity of the bladder actomyosin ATPase by calmodulin interaction (Tokuno et al. 1993; Matsushima et al. 1997; Moriya et al. 1999; Madersbacher and Mürtz 2001; Andersson 2002). However, there might be additional intracellular targets to explain the beneficial pharmacodynamic net effect of propiverine in man.

There is many evidence that protein kinase C (PKC) mediated signal transduction pathways are involved into the regulation of the detrusor smooth muscle contractions (1) by auto-facilitation of the pre-synaptic muscarinic (M1) mediated acetylcholine release (Somogyi et al. 1996), (2) by controlling the purinergic components of the signal cascade via Ca^{2+} channels (Lin et al. 1998; Liu and Lin-Shiau 2000), (3) by modulation of the intracellular Ca^{2+} independent myosin light chain phosphorylation (Yoshimura and Yamaguchi 1997), (4) by regulation of the cell-to-cell signal transmission via gap junctions (Lacaz-Viera 2000), and (5) by expression of the nerve growth factor (NGF) a molecular trigger of the afferent nervous control of detrusor contractions (Tanner et al. 2000; Morrision et al. 2002).

Therefore, we hypothesized that inhibition of the bladder PKC might be a target in the pharmacological treatment of overactive bladder by inhibition of PKC-mediated smooth muscle contractions and muscle excitability caused by changes in intercellular coupling and modulation of the afferent sensoric activation. We will show, that propiverine additionally to its known multiple non-cholinergic actions may be bladder relaxing by inhibition of the PKC in the urinary bladder.

2. Investigations and results

Chronic treatment of Lewis rats with propiverine in increasing oral doses between 0.6 and 60 mg/kg for five days resulted in translocation of the membrane bound PKC of the urinary bladder. The active, membrane-bound PKC decreased by about 60% accompanied by a corresponding increase of the soluble form of the enzyme. The no effect dose of propiverine has been 0.6 mg/kg. 2 mg/kg and more caused significant differences. The magnitude of changes after propiverine treatment on both forms of the hepatic PKC was markedly lower. The no effect dose on the membrane-bound form was 2.0 mg/kg; a significant elevation of the soluble variant has been observed only with the highest dose of 60 mg/kg propiverine (Table).

In our *in vitro* experiment, propiverine inhibited the membrane-bound PKC of rat bladder in a concentration depen-

Table 1: Effects of propiverine on the membrane-bound and soluble form of the protein kinase C (activities in nmol × min/mg) of urinary bladder and liver after pre-treatment of rats for 5 days

	Urinary bladder		liver	
	membrane-bound	soluble	membrane-bound	soluble
Control	3.06 ± 0.36	2.25 ± 0.25	1.58 ± 0.23	2.23 ± 0.16
0.6 mg/kg	3.52 ± 0.53	2.30 ± 0.30	1.52 ± 0.16	2.27 ± 0.19
2.0 mg/kg	1.68 ± 0.16**	3.57 ± 0.30*	1.40 ± 0.19	2.36 ± 0.32
6.0 mg/kg	1.48 ± 0.25**	3.48 ± 0.43*	0.82 ± 0.10**	2.37 ± 0.22
60 mg/kg	1.24 ± 0.19**	3.49 ± 0.29*	0.81 ± 0.05**	3.48 ± 0.19**

* $p < 0.05$, ** $p < 0.01$ compared to the respective controls
Means ± SD are given. The statistical evaluation was done with the Mann-Withney U-test.

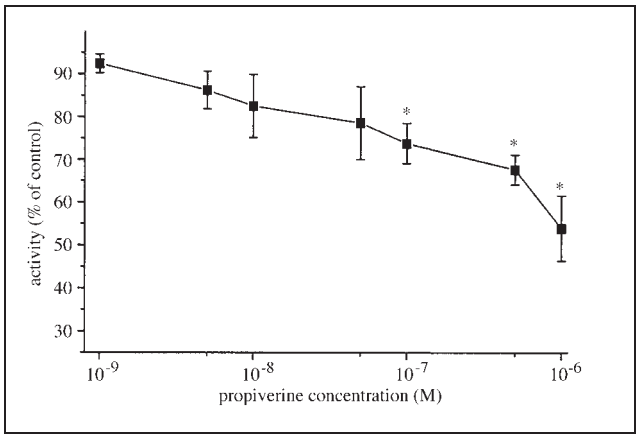

Fig.: Activity of the membrane-bound protein kinase C of the urinary bladder of rats after incubation with propiverine. Means ± SD of 5 experiments are given. The statistical assessment was done with the Mann-Withney U-test. * $p < 0.05$

dent manner (Fig. 1). 100 nM has been the lowest concentrations that caused significant competition. The N-oxidized metabolites of propiverine (M5, M6) and atropine were without any effect in concentrations between 1 nM and 1 μM. High concentrations of the despropyl metabolite (M2) inhibited the PKC only slightly (data not shown).

3. Discussion

We have clearly shown that the bladder spasmolytic drug propiverine translocates the membrane-bound PKC in partially purified cell fractions obtained from the urinary bladder and the liver of rats after chronic pretreatment with daily doses of 0.6 mg/kg and above. 0.6 mg/kg is the medium therapeutic dose of propiverine in patients with detrusor hyperactivity. Furthermore, the enzyme has been also inhibited in vitro with concentrations that were similar in value to the therapeutic serum concentrations in man (Franke et al. 2000). Therefore, we believe that inhibition of the bladder PKC may contribute to the overall pharmacodynamic effect of the drug.

Propiverine is a muscarinic receptor blocking agent with some selectivity for the M_3 receptor subtype (Andersson 2002). However, it exerts also non-cholinergic effects such as L-type calcium channel antagonism and calmodulin interaction associated with inhibition of the myosin light chain kinase (Tokuno et al. 1993; Matsushima et al. 1997; Moriya et al. 1999; Madersbacher and Mürtz 2001). Inhibition of the PKC which seems to influence bladder contraction independent on the neurogenic transduction pathway is considered to be a further beneficial mechanism to overcome the so-called "atropine resistance" in patients with detrusor hyperactivity. Propiverine in our in-vitro study has been inhibitory on the bladder PKC in two-fold to three-fold lower concentrations as necessary to influence the myosin light chain kinase (Matsushima et al. 1997).

According to the findings of Yoshimura and Yamaguchi (1997), there is a calcium independent contraction which seems to be mediated by activation of the PKC coupled with agonist stimulation of the muscarinic receptor. Furthermore, Lin et al. (1998) and Liu and Lin-Shiau (2000) found that L-type calcium channels are activated through phosphorylation by PKC. After incubation with the PKC activator β-phorbol-12,13-dibutyrate, an increase in strength of the mouse, rabbit and human bladder strip contractions was measured. This effect was susceptible to antagonism with the PKC inhibitors staurosporine, bisindolylmaleinimide and H-7. Bisindolylmaleinimide analogues are widely used as selective inhibitors of the PKC (Toullec et al. 1991; Coultrap et al. 1999). Lazareno et al. (1998) described that these compounds are inhibitors of the PKC in concentrations of 10–100 nM.

Furthermore, the PKC seems to be a key factor in the regulation of the cell-to-cell signal transmission by controlling the opening of gap junctions (Lacaz-Viera 2000). The PKC inhibitor H-7 inhibited completely the tight junction opening in response to basolateral calcium withdrawal. The PKC is also known to be auto-facilitatory on the pre-synaptic muscarinic (M1) mediated acetylcholine release (Somogyi et al. 1996). Finally, activation of the PKC in detrusor muscle cells by phorbol esters *in vitro* leads to expression of the NGF which is elevated in the bladders of patients with idiopathic detrusor hyperactivity. NGF is a molecular trigger in the afferent nervous control and antibodies against NGF or its receptor prevent urinary frequency and unstable contraction (Tanner et al. 2000; Morrision et al. 2002). On the contrary, however, recent data have shown that phospholipase C (PLC) – PKC cascades may be activated by muscarinic receptors in rat bladder without being crucial for induction of the muscle contraction.

In conclusion, the bladder spasmolytic drug propiverine was identified to be an inhibitor of the protein kinase C. Whether this contributes to the non-cholinergic control of contractions in hyperactive detrusor smooth muscle cells needs further investigation.

4. Experimental

4.1. Animals and pretreatment

Inbred male LEW 1A rats weighing 200–290 g were obtained from the central animal house of the University of Greifswald and adapted in groups of four animals in polycarbonate cages (bedding ssniff, type lignocel, 3–4 fibres, Soest, Germany) to the conditions of our life-island system (Flufrance, type A 110, Wissous, France) for at least two weeks with free

access to standard diet (ssniff, type R-Z, extruded, Soest, Germany) and acidified tap water. Light was on between 7 AM and 7 PM. The room temperature was set to 25 ± 1 °C. The air of the system has been replaced 8 times per hour.

In the morning of 5 consecutive days, the animals were treated with tap water (control) or propiverine in doses of 0.6; 2.0; 6.0; and 60 mg/kg body weight using an intestinal gauge. The administrations volume has been 5 ml/kg.

4.2. Activity of protein kinase C

After overnight fasting, the animals were sacrificed by cervical dislocation and decapitation in the morning of at the 6th day. The urinary bladder and the liver were dissected for partial purification of the soluble and membrane-bound PKC with DEAE-cellulose columns according to the method of Wang and Friedman (1990). PKC enzyme activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP (Amersham Biotech, Freiburg, Germany) into lysine-rich histone (type III-S, Sigma, Deisenhofen, Germany). The assay mixture (0.25 ml, pH 7.5) consisted of (final concentrations) 5 mM NaCl, 0.025 mM EGTA, 24 mM Tris-HCl, 0.015% 2-mercaptoethanol, 30 µg/ml leupeptin, 0.01 mM PMSF, 400 µg/ml histone, 1.2 mM $CaCl_2$, 10 µg/ml phospatidyl serine, 5 µg/ml 1,2-diolein, 10 mM magnesium acetate and 29.85 µM [γ-^{32}P]-ATP. The samples were pre-incubated at 30 °C for 5 min. The enzyme reaction was started by adding of 25 µl of the column eluates and incubated at 30 °C for 5 min. The reactions were terminated with 1 ml trichloroacetic acid (25% with 2% pyrophosphate). The mixtures were filtered through nitrocellulose acetate membranes (pore diameter 0.45 µm, Schleicher & Schüll, Dassel, Germany). After washing the filters with 10 ml tricloroacetic acid (10%, with 1% pyrophosphate), the radioactivity retained on the filter was measured by liquid scintillation counting (LKB-Wallac, Turku, Finland) with a toluene-containing scintillator (0.5% PPO, 0.05% POPOP) (Bailey et al. 1972). The enzyme assays were done in duplicates with reference to samples without phospatidyl serine and 1,2-diolein. Activities were expressed as nmol phosphate/mg protein/min. Protein concentrations of the column eluates were measured with bovine serum albumin as standard according to the method of Bradford (1976).

For *in vitro* competition assays, partially purified column eluates of the membrane-bound PKC fraction were incubated at 30 °C for 10 min with propiverine and its despropylated metabolite (M2), with their N-oxides (M5, M6) and with atropine in concentrations between 1 nM and 1 µM. The enzyme activities were measured as described above.

4.3. Statistical evaluation

Means ± standard deviation (SD) are given. Differences were assessed with the U-test according to Mann and Withney.

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