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Novel effect of 2-aminoethoxydiphenylborate through inhibition of calcium sensitization induced by Rho kinase activation in human detrusor smooth muscle ☆

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ABSTRACT

Since the introduction of 2-aminoethoxydiphenylborate (2-APB) as a membrane permeable modulator of inositol (1,4,5)-trisphosphate receptors, subsequent studies have revealed additional actions of this chemical on multiple Ca^{2+}-permeable ionic channels in the plasma membrane. However, no reports have yet examined 2-APB as a modulator targeting contractile machinery in smooth muscle, independent of Ca^{2+} mobilization, namely Ca^{2+} sensitization. Here, we assessed whether or not 2-APB affects intracellular signaling pathways of Ca^{2+} sensitization for contraction using α-toxin permeabilized human detrusor smooth muscle. Although contractions were induced by application of Ca^{2+}-containing bath solutions, 2-APB had little effect on contractions induced by 1 μM Ca^{2+} alone but significantly reversed the carbachol-induced augmentation of Ca^{2+}-induced contraction in the presence of guanosine triphosphate (carbachol-induced Ca^{2+} sensitization). The rho kinase inhibitor Y-27632 and protein kinase C inhibitor GF-109203X also reversed the carbachol-mediated Ca^{2+} sensitization. Additional application of 2-APB caused a small but significant further attenuation of the contraction in the presence of GF-109203X but not in the presence of Y-27632. Like carbachol, the rho kinase activator; sphingosylphosphorylcholine, protein kinase C activator; phorbol 12,13 dibutyrate, and myosin light chain phosphatase inhibitor; calyculin-A all induced Ca^{2+} sensitization. However, the inhibitory activity of 2-APB was limited with sphingosylphosphorylcholine-induced Ca^{2+} sensitization. This study revealed a novel inhibitory effect of 2-APB on smooth muscle contractility through inhibition of the rho kinase pathway.

1. Introduction

2-aminoethoxydiphenylborate (2-APB) was first introduced as a membrane-permeable inhibitor of inositol (1,4,5)-trisphosphate (IP_3) receptors for suppressing Ca^{2+} release from intracellular Ca^{2+} stores (Maruyama et al., 1997). Accordingly, numerous reports have been published to support 2-APB as an IP_3 receptor inhibitor and to clarify the intracellular Ca^{2+} signaling cascade. However, several studies have revealed a number of additional effects; for example, 2-APB also blocks transient receptor potential (TRP) channels such as TRPM7 (Ratz and Berg, 2006; Hu et al., 2004; Hamaguchi et al., 2008), store-operated Ca^{2+} channels (Bootman et al., 2002), and connexin-based gap junction channels (Harks et al., 2003). Although there are varieties of 2-APB effects, all of these effects are related to Ca^{2+} mobilization (Ca^{2+} dependent pathway) as mentioned above leading to suppress smooth muscle contraction. On the other hand, there is no report investigating the effect of 2-APB on Ca^{2+} sensitization (Ca^{2+} independent pathway).

A rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is essential for the initiation of smooth muscle contraction (Ca^{2+}-dependent pathway) and the key determinant of cross-bridge regulation (Somlyo and Somlyo, 1994). In the presence of calmodulin, intracellular Ca^{2+} activates Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK), which in turn increases the phosphorylation of 20 kD myosin light chain (MLC_{20}), leading to contraction (see review Horowitz et al., 1996). Phosphorylation of MLC_{20} is further promoted through inhibition of myosin light chain phosphatase (MLCP), thereby potentiating force development of contractile proteins at constant $[Ca^{2+}]_i$, referred to as Ca^{2+}-sensitization (Ca^{2+} independent pathway) through the

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activation of two major pathways, namely rho-kinase (ROK) and protein kinase C (PKC) (Himpens et al., 1988; Kitazawa et al., 1991; Shahab et al., 2012a, 2012b; Somlyo and Somlyo, 2003). Smooth muscle thus contracts in Ca^{2+}-dependent and Ca^{2+}-independent manners through activation and inactivation of MLCK and MLCP activities, respectively. Furthermore, it seems likely that some thin filament-linked modulations are cooperating in parallel (Marston and Smith, 1985; Smith et al., 1987; Kajioka et al., 2012; Kim et al., 2008). Therefore, drugs permeable across the plasma membrane may affect smooth muscle contractility through mechanisms liked with thick and/or thin filaments.

The one of the reagent advantages in 2-APB is also membrane penetrable, regardless of its intracellular effect, however this may be the reason why the effect of 2-APB had not been investigated on membrane permeabilized tissues until Durlu-Kandilci and Brading (2006) reported that 2-APB attenuates contractions of rat detrusor smooth muscle strips which were permeabilized with β-escin. Since $[Ca^{2+}]_i$ was controlled in their experiments, it is considered that 2-APB also affects Ca^{2+}-independent pathways of smooth muscle contraction. However, this effect was irreproducible in β-escin permeabilized smooth muscle strips obtained from guinea-pig detrusor and taenia caeci; as such, whether or not this drug affects mechanisms other than IP_3 receptors and ionic channels remains unclear. In the present study, we thus examined the effects of 2-APB on the force development in human detrusor strips permeabilized with α-toxin, which more efficiently preserves receptor–effector pathways than β-escin (Nishimura et al., 1988; Takahashi et al., 2004; Wu et al., 1995).

2. Materials and methods

2.1. Tissue specimens

Smooth muscle tissue was obtained from urinary bladders of human patients (mean age: 68 ± 2.5 years) who had undergone radical cystectomy due to bladder cancer. No patients had invasive bladder cancer. Specimens were extracted from tumor-free regions by gently excising the smooth muscle tissue from the urinary bladder and immediately placing the specimens into ice-cold physiological salt solution.

Written informed consent was obtained from all patients. The protocol of this study was approved by the Ethical Committee of Graduate School of Medical Sciences, Kyushu University.

2.2. Smooth muscle preparation and α-toxin permeabilization

Under a dissecting microscope, the mucosa and connective tissues were gently removed. The smooth muscle bundle was isolated and cut longitudinally into small strips measuring 200–300 μm in width and 3–4 mm in length, and a longitudinal slit was made along each strip. The strips were then placed into relaxing solution for 1–2 min to remove extracellular Ca^{2+} and permeabilized in relaxing solution containing 5000 U/ml α-hemolysin (toxin) from *Staphylococcus aureus* for 1 h, as previously described (Shahab et al., 2012a, 2012b).

The permeabilized detrusor smooth muscle strips were mounted horizontally between two tungsten wires, each of which was connected to a force transducer (UL2 Minebea Co. Ltd., Osaka, Japan) on perspex disc in 100 μl relaxing solution. A resting tension of 0.1 g was applied for 1 h. All permeabilized strips were treated with Ca^{2+} ionophore A 23187 (1 μM) for 30 min. The experiment was carried out after pre-incubation with 1 μM xestospongin C and 1 μM thapsigargin for 30 min and with 1 μM CPA present in all solutions after permeabilization (Shahab et al., 2012a). All experiments were performed at room temperature (25 °C) within 24 h after the radical cystectomy.

2.3. Data analysis and statistical procedures

Data were obtained from computerized data acquisition system (MacLab; Analog Digital Instruments, Sydney, Australia, and Apple Corp., Sunnyvale, CA, USA) and presented as mean ± standard error of the mean (S.E.M). The activation curve in Fig. 2 was drawn using the following equation:

$$\text{Tension}(\%) = \frac{\text{Tension}_{\max}}{(1+(EC_{50}/[Ca^{2+}]_i)^{nH})}$$

The EC_{50} is the concentration of Ca^{2+} that activated the relative value of the contraction response to half, and nH is the Hill coefficient. Statistical analyses were performed using an independent Student's t-test with SPSS software version 19 (IBM, New York, USA). $P<0.05$ was considered to be statistically significant.

2.4. Drugs, chemical reagents, and other materials

α-hemolysin (toxin) from Staphylococcus aureus, carbachol, guanosine 5′-triphosphate (GTP), sphingosylphosphorylcholine (SPC), cyclopiazonic acid (CPA), phorbol 12,13-dibutyrate (PDBu), and 2-Aminoethyl diphenyl borate (2-APB) were obtained from Sigma (St. Louis, MO, USA). Y-27632 ((R)-(+)-trans-N(4-pyridil)-4-(1-aminoethyl)-cyclohexanecarboximide dihydrochloride, monohydrate), GF-109203X (bisindolylmaleimide I), A-23187, and xestospongin C were obtained from Calbiochem, (La Jolla, CA, USA). calyculin A was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Physiological salt solution (PSS) contained 123 mM NaCl, 15.5 mM $NaHCO_3$, 11.5 mM D-glucose, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 1.2 mM KH_2PO_4 gassed with 5% CO_2 and 95% O_2 (pH 7.4). Relaxing solution was composed of 100 mM potassium methansulphonate, 20 mM tris-maleate, 10 mM ethyleneglycol-bis (β-aminoethylether)-N′,N′,N′,N′ tetra acetic acid (EGTA), 10 mM creatinine phosphate, 3.38 mM $MgCl_2$, and 2.2 mM Na_2 adenosine triphosphate at pH 6.8. Activating solutions containing the indicated concentration of free Ca^{2+} were created by adding an exact amount of $CaCl_2$ to the relaxing solution using a Ca^{2+}–EGTA binding constant of 10^6/M.

3. Results

3.1. Inhibition of carbachol-mediated Ca^{2+} sensitization by 2-APB

Although 2-APB is known to affect a wide range of cellular mechanisms, the quite high concentration of 2-APB (30–100 μM) has been accepted for investigation (Maruyama et al., 1997; Ratz and Berg, 2006; Hu et al., 2004; Durlu-Kandilci and Brading, 2006). In agreement with previous studies, our preliminary experiments indicated that the inhibitory action of 2-APB on carbachol-induced Ca^{2+} sensitization was observed beginning at 10 μM in a concentration-dependent manner with an IC_{50} of 88 μM (Supplemental Fig. S1). Therefore, 100 μM 2-APB close to that IC_{50} was applied throughout the present experiments.

First, the effects of 2-APB were examined on force development induced by only an increase in $[Ca^{2+}]_i$. 2-APB at 100 μM induced little change in the contractile response to 1 μM $[Ca^{2+}]_i$ ($N=4$; $n=8$, Fig. 1A). Subsequently, the effect of 2-APB (100 μM) was examined on carbachol-induced Ca^{2+} sensitization at fixed 1 μM $[Ca^{2+}]_i$. Since muscarinic receptors are coupled with G-proteins, 100 μM GTP was added prior to the application of 10 μM carbachoí. Stimulation of muscarinic receptors by carbachol in the presence of GTP further increased the tension at fixed 1 μM $[Ca^{2+}]_i$ (Ca^{2+} sensitization) (530.5% ± 60.3% $N=6$, $n=18$). The application of 100 μM 2-APB significantly decreased this carbachol-mediated