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A model of bidirectional regulation induced by a combination of one stimulatory and one inhibitory agent on gastric smooth muscle contraction

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In this paper, we proposed a model of counterbalance resulting from bidirectional regulation on gastric smooth muscle contraction in the absence of nervous and endocrine system control. To verify the propose, we suggested that a combination of one stimulatory and one inhibitory drug should produce bidirectional regulation on the contractility of gastric smooth muscle and myosin activity, depending on the contractile state of the gastric smooth muscle and myosin phosphorylation extent, regardless of the individual mechanism of drug action. We selected two stimulatory drugs, that is, hesperidin and emodin, and three inhibitory drugs, that is, berberine, synephrine, and quercetin, using them in stimulatory-inhibitory drug pair. Our assay supports the model we proposed. The common characteristics of these drug combinations demonstrate that inhibitory effect is observed when smooth muscle strip is present in a relatively high contractile state and myosin is fully phosphorylated, and that stimulatory effect is observed when smooth muscle strip is in a relatively low contractile state and myosin is partially phosphorylated. Regardless of the individual mechanism of drug action, the combination of one stimulatory and one inhibitory agent showed the characterization of counterbalance resulting from bidirectional regulation. This model implies the presence of counterbalance in cellular level maintained by bidirectional regulation in gastric smooth muscle, and the combination of one stimulatory and one inhibitory drug provides the information for the potential therapy of gastric smooth muscle contractility related disorders.

Keywords: Bidirectional regulation, smooth muscle contractility, dynamic counterbalance, myosin, Mg^{2+}-ATPase activity, phosphorylation.

INTRODUCTION

Smooth muscle contractility is mainly regulated by phosphorylation of the 20 kDa myosin light chains (LC_{20}), which is controlled by the opposing activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) (Ihara and Macdonald, 2007). Besides MLCK, other modulators, e.g., Rho-kinase, protein kinase C (PKC), and caldesmon are also involved in regulating the myosin function. How these modulators are related and integrated in the regulation of smooth muscle contraction remains unclear. From an alternative viewpoint, we may notice that bidirectional regulation (BR) can be summarized as a common characterization of these modulators in regulating the activities of smooth muscle myosin.

BR can be categorized into the following three subtypes: (1) BR results from phosphorylation and dephosphorylation of same substrate, e.g., myosin light chain phosphorylation by MLCK and dephosphorylation

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Abbreviations: BR, Bidirectional regulation; **GSMS**, gastric smooth muscle strips; **SIDP**, stimulatory-inhibitory drug pair; **CDPM**, Ca^{2+}-dependent phosphorylation of myosin; **PAGE**, polyacrylamide gel electrophoresis.

by phosphatase produces stimulatory and inhibitory effect respectively on myosin activity (Ihara and Macdonald, 2007; Somlyo and Somlyo, 1994). (2) BR results from phosphorylation of different substrates. For example, phosphorylation of MLCK by PKC inhibits myosin activities (Nishikawa et al., 1985) and phosphorylation of myosin phosphatase by PKC (Pfitzer, 2001) activates myosin activities. (3) BR results from regulatory protein binding to both myosin and actin. For example, calponin stimulates phosphorylated myosin activity in the absence of actin by direct interaction with myosin, and inhibits the activity in the presence of actin (Winder and Walsh, 1990; Lin et al., 1993). We have mentioned three subtypes of intracellular BR rather than illustrating all of the BR in regulating myosin activities of smooth muscle. The evidence of a BR can also be described as smooth muscle, is maintained in a state neither over-contracted nor over-relaxed in normal conditions.

Based on our viewpoint that diverse BR is integrated in modulating gastric smooth muscle contraction, and on the observation that the combination of emodin (stimulatory) and quercetin (inhibitory) produces BR on myosin function of smooth muscle (Zhang et al., 2006), we propose a model of counterbalance resulting from bidirectional regulations in modulating gastric smooth muscle contraction in the absence of the control of nervous and endocrine system. To verify the propose, we suggest that combination of any one of stimulatory and any one of inhibitory drug produces BR on contractility of gastric smooth muscle, depending on gastric smooth muscle contraction, and regardless the individual mechanism of drug action.

We selected two indexes to observe modulation of gastric smooth muscle contraction. Firstly, gastric smooth muscle contractility were measured respectively in a relatively high contractile state and in a relatively low contractile state by adjusting the Ca^{2+} concentration in the assay, using gastric smooth muscle strips (GSMS). Since GSMS contains all the intracellular kinase, phosphatase and other modulators, the contractility of GSMS is considered to be integrated from the interaction between myosin with those modulators. Secondly, Mg^{2+}-ATPase activity and phosphorylation of myosin light chains are chosen as molecular level indexes because they are positively related to smooth muscle contractility (Kenney et al., 1990).

We set a criterion for selecting drugs in our study, that is, when used alone, the drug selected produces a stimulatory effect or an inhibitory effect on the function of smooth muscle. We used stimulatory-inhibitory drug pair (SIDP) in the assay. Our preliminary assay indicated that hesperidin and emodin showed a stimulatory effect respectively, and berberine, synephrine, and quercetin showed an inhibitory effect, respectively. We used them in SIDP, that is, hesperidin with berberine, hesperidin with synephrine, hesperidin with quercetin, emodin with berberine, and emodin with synephrine.

The assay is to verify the BR model we proposed via showing whether presence of the common characterization of SIDP produced effect on smooth muscle contraction rather than revealing individual mechanisms of drug action.

MATERIALS AND METHODS

25 μmol/L of hesperidin, emodin, berberine, synephrine, and quercetin were used in determination phosphorylation and Mg^{2+}-ATPase activity of myosin, respectively. SIDP is arranged in the foregoing assay. The BR data from emodin with quercetin are not shown but reported previously (Zhang et al., 2006).

Protein purification

Myosin and MLCK used in the assay were purified from fresh chicken gizzard smooth muscle using methods described previously (Tang et al., 2010).

Myosin phosphorylation and Mg^{2+}-ATPase activity determination

The procedures for determining Ca^{2+}-dependent phosphorylation of myosin (CDPM) and Mg^{2+}-ATPase activities of phosphorylated and unphosphorylated smooth muscle myosin were assayed in 10% glycerol polyacrylamide gel electrophoresis (PAGE) as described previously (Yang et al., 2003). Fully phosphorylated myosin and partially phosphorylated myosin were obtained by adding MLCK to the final concentrations of 0.02 and 0.0002 μmol/L, respectively in the assay. 4 and 0.4 μmol/L myosin were used in phosphorylation and Mg^{2+}-ATPase activity determination, respectively.

Preparation of gastric muscle strips of rats

GSMS was obtained from Male Sprague-Dawley rats (200 to 300 g). GSMS (2 cm long, 0.4 cm wide) were suspended in 20 ml chambers containing Krebs' solution (Tan et al., 2006). High contractile state of GSMS was assayed in Krebs' solutions containing 5.0 mmol/L $CaCl_2$, and low contractile state of GSMS was assayed in Krebs' solutions containing 1.25 mmol/L $CaCl_2$.

Calculation

Scoin Image software was used to scan the density and size of the phosphorylated MLC_{20} and to calculate the percentage of phosphorylated MLC_{20} in the total MLC_{20}. For phosphorylation extent and myosin Mg^{2+}-ATPase activities, both of fully and patially phosphorylated myosin controls were calculated as 100% respectively, and all the data of SIDP were the relative value calculated from SIDP/control. Data were expressed as $\bar{X}$ ±s, and the Student's t-test was used to evaluate the significance of differences.

RESULTS

The establishment of BR model

The model of counterbalance resulting from integration of

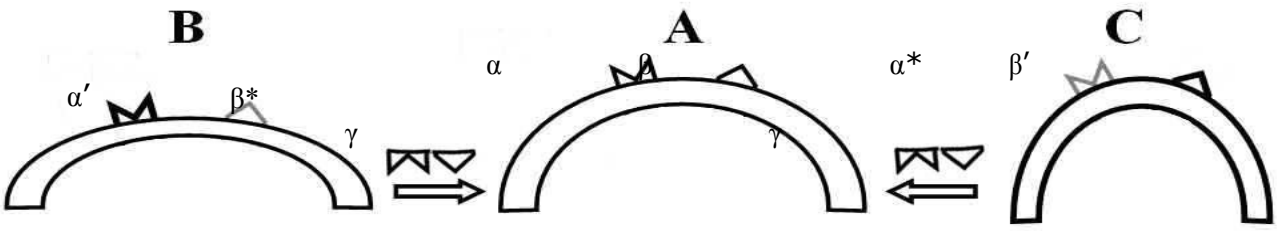

Figure 1. A model of counterbalance via BR induced by SIDP. α and β represent nonselective receptors (representing any cellular target which can respond to drug action) to stimulant and inhibitor respectively. α' and α* represent higher and lower potency of those nonselective receptors respectively and respond to stimulants. β' and β* represent higher and lower potency of those nonselective receptors respectively and respond to inhibitors. γ represents SIDP. Panel A represents normal response (α and β) of smooth muscle cell to SIDP in normal contractile state; Panel B represents high response (α') of smooth muscle cell to stimulatory effect resulting from SIDP while smooth muscle in low contractile state; C represents high response (β') of smooth muscle cell to inhibitory effect resulting from SIDP while smooth muscle in high contractile state.

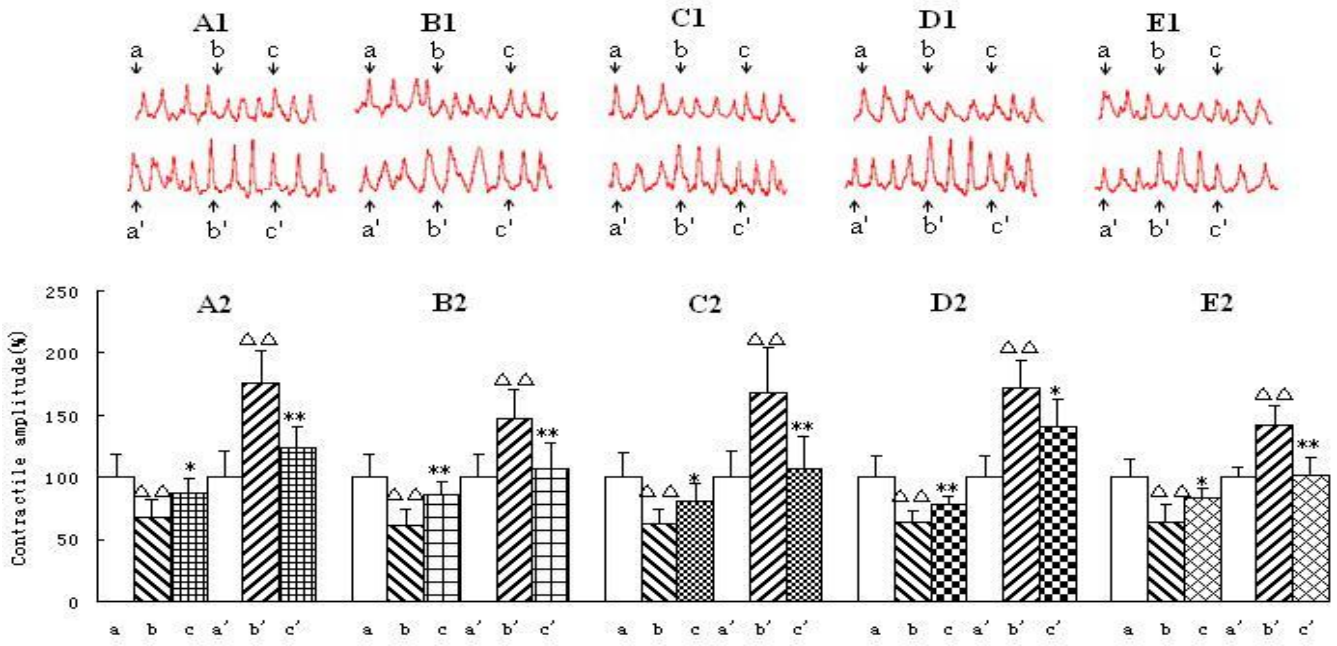

Figure 2. Representative records of BR induced by SIDP on the contractility of GSMS in different contractile states. The records from A1 to E1 represent the isometric response to SIDP. Data from A2 to E2 are the s.e. means of calculated data from 6 experiments including the representative records in A1 to E1, respectively. The results obtained with an assay condition of 2.5 mmol/L Ca^{2+} (normal) are marked with (a) and (a'), 2 mmol/L Ca^{2+} (low) with (b) and (c), and 5 mmol/L Ca^{2+} (high) with (b') and (c'). The effects produced by SIDP are marked (c) and (c'). SIDP is described as follows: 100μmol/L hesperidin + 25 μmol/L berberine (A1, A2); 100 μmol/L hesperidin + 50 μmol/L synephrine (B1, B2); 50 μmol/L hesperidin + 12.5 μmol/L quercetin (C1, C2); 25 μmol/L emodin + 6.25 μmol/L berberine (D1, D2); 25 μmol/L emodin + 6.25 μmol/L synephrine (E1, E2). △△p<0.01 vs. normal Krebs' solution, *p<0.05, **p<0.01 vs. different calcium concentration of Krebs' solution.

BR is described in Figure 1. In low contractile state (Figure 1B) (α'), SIDP produces a stimulatory effect; and in high contractile state (Figure 1C) (β'), SIDP produces an inhibitory effect. Regardless of the individual mechanism of action, SIDP facilitates smooth muscle cell both in low contractile state (Figure 1B) and high contractile state (Figure 1C) back to normal state (Figure 1A), that is., BR produced by SIDP forms counterbalance of gastric smooth muscle contraction.

To identify the model we proposed , we selected GSMS contractility, myosin phosphorylation extent and myosin Mg^{2+}-ATPase activities as indexes in the assay.

Effect of SIDP on the contractility of GSMS

Figure 2 A to E shows that all the 5 pairs of SIDP produce significant stimulatory effects on GSMS in the low

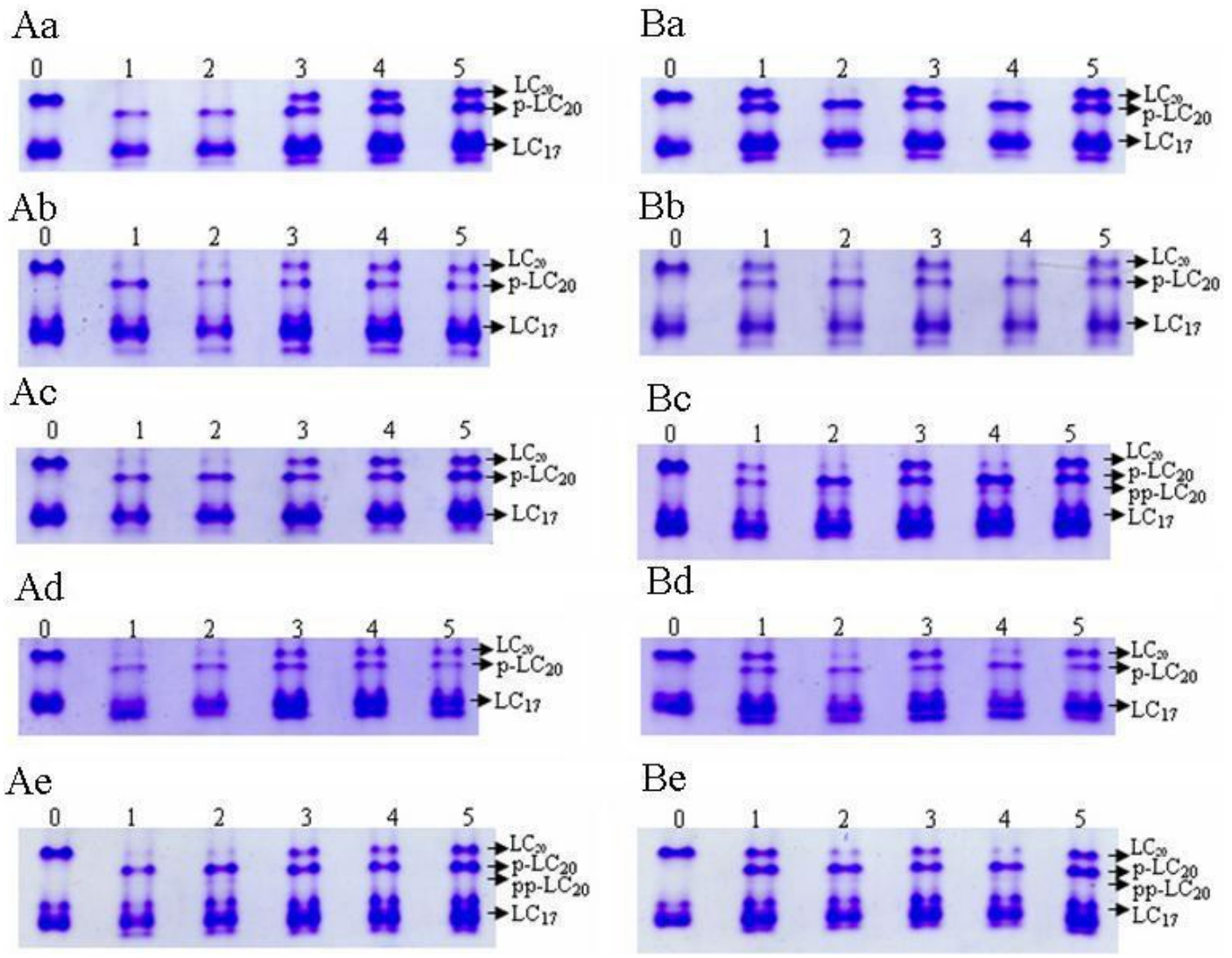

Figure 3. Representative records of BR induced by SIDP on myosin phosphorylation. Representative records of fully phosphrylated myosin in listed from penal Aa to panel Ae, and representative records of from partially phosphrylated myosin is listed from penal Ba to panel Be. In both panel A and panel B, lane 0 represents unphosphorylated myosin; lane 1 represents CDPM; lane 4 represents hesperidin+berberine (a), hesperidin+synephrine (b), hesperidin+quercetin (c), emodin+berberine (d) and emodin+synephrine (e) respectively; LC_{20} represents unphosphorylated MLC_{20}; p-LC_{20} represents mono-phosphorylated MLC_{20}; pp-LC_{20} represents di-phosphorylated MLC_{20}; LC_{17} represents 17 kDa myosin essential light chains.

contractile state (c), ($p < 0.05$ and $p < 0.01$), and produce significant inhibitory effects on GSMS in the high contractile state (c'), ($p < 0.01$). It should be noted that BR produced by SIDP is observed in certain condition, that is, the dose ratio of stimulatory/inhibitory agent should be within a certain range. The data for selecting unsuitable dose ratio of SIDP is not shown.

BR induced by SIDP on myosin phosphorylation

Figure 3 and Table 1 show that BR is induced by the 5 pairs of SIDP on myosin phosphorylation. Compared to the controls (fully phosphorylated myosin, panel Aa to Ae, lane 1), the 5 pairs of SIDP (Aa to Ae, lane 4) significantly inhibit the phosphorylation; and compared to the controls (partially phosphorylated myosin, panel Ba-Be, lane 1), the 5 pairs of SIDP (Ba to Be, lane 4) significantly increase the phosphorylation.

BR induced by SAIA on myosin Mg^{2+}-ATPase activities

Compared to their controls, the 5 pairs of SIDP significantly inhibit the Mg^{2+}-ATPase activities of fully

Table 1. BR induced by SAIA on myosin phosphorylation.

Fully phosphorylated myosin (part A) (%)	Partially phosphorylated myosin (part B) (%)
Aa-lane1 CDPM control, (100.0±1.4)	Ba-lane 1 CDPM control, (100.0±3.3)
Aa-lane 4 hesperidin+berberine, (57.4±1.4)**	Ba-lane 4 hesperidin+berberine, (137.6±8.6)**
Ab-lane 1 CDPM control, (100.0±0.5)	Bb-lane 1 CDPM control, (100.0±3.4)
Ab-lane 4 hesperidin+synephrine, (69.0±0.6)**	Bb-lane 4 hesperidin+synephrine, (149.9±8.1)**
Ac-lane 1 CDPM control, (100.0±3.0)	Bc-lane 1 CDPM control, (100.0±5.6)
Ac-lane 4 hesperidin+quercetin, (64.4±3.1)**	Bc-lane 4 hesperidin+quercetin, (147.7±9.3)**
Ad-lane 1 CDPM control, (100.0±1.8)	Bd-lane 1 CDPM control, (100.0±2.6)
Ad-lane 4 emodin+berberine, (62.4±5.5)**	Bd-lane 4 emodin+berberine, (132.7±11.7)**
Ae-lane 1 CDPM control, (100.0±4.1)	Be-lane 1 CDPM control, (100.0±1.7)
Ae-lane 4 emodin+synephrine, (72.7±1.9)**	Be-lane 4 emodin+synephrine, (143.7±3.7)**

The data are expressed as s.e. means of calculated value from 6 experiments including the representative records in Figure 3.
** $p<0.01$ vs CDPM control respectively in both part A and part B.

Table 2. BR induced by SAIA on myosin Mg^{2+}-ATPase activity.

Fully phosphorylated myosin (part A) (%)	Partially phosphorylated myosin (part B) (%)
CDPM control, (100.0±4.1)	CDPM control, (100.0±8.8)
Hesperidin+berberine, (77.9±2.8)**	Hesperidin+berberine, (127.0±13.4)**
CDPM control, (100.0±2.1)%;	CDPM control, (100.0±9.8)
Hesperidin+synephrine, (72.9±2.5)**	hesperidin+synephrine, (124.3±5.7)**
CDPM control, (100.0±5.6)%;	CDPM control, (100.0±8.2)%;
Hesperidin+quercetin, (80.4±5.2)**	Hesperidin+quercetin, (134.1±7.4)**
CDPM control, (100.0±3.1)%;	CDPM control, (100.0±9.0)%;
Emodin+berberine, (82.5±7.7)**	Emodin+berberine, (120.0±9.2)**
CDPM control, (100.0±6.3)%;	CDPM control, (100.0±8.1)
Emodin+synephrine, (72.4±4.5)**	Emodin+synephrine, (128.4±5.2)**

** $p<0.01$ vs CDPM control respectively in both part A and part B. The data are illustrated as s.e. means of calculated value of 6 experiments.

phosphorylated myosin (Table 2 part A), but significantly increase the Mg^{2+}-ATPase activities of partially phosphorylated myosin (Table 2 part B).

DISCUSSION

The drugs used in this study have various biological effects (Garg et al., 2001; Huang et al., 1991; Shirwaikar et al., 2006; Haaz et al., 2006; Garcia-Saura., 2005). However, our study did not focus on the different mechanisms resulting from these drug combinations on smooth muscle contractility and myosin function. Instead, we have tried to utilize SIDP to verify the BR model we proposed.

Our assay supports the BR model we proposed. The common characterization of SIDP from these combinations is that it produces stimulatory effect on GSMS in low contractile state, and increases Mg^{2+}-ATPase activity and phosphorylation of partially phosphorylated myosin; and SIDP produces inhibitory effect on GSMS in high contractile state and inhibits Mg^{2+}-ATPase activity and phosphorylation of fully phosphorylated myosin; These results suggests that BR induced by SIDP depends on the contractile state and myosin function state of the smooth muscle, regardless of individual mechanism of drug action.

The BR model provides the following alternative viewpoint. Firstly, the counterbalance is automatically achieved by BR induced by SIDP in modulating smooth muscle contraction in the absence of control from nervous and endocrine system. Secondly, this BR model suggests an alternative viewpoint for preclinical drug candidate test for the potential therapy of gastric smooth muscle contractility disorders by using SIDP. However, before public acceptation of the BR model, it should be tested in different Lab, and in different type of smooth muscle.

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