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Adipokines as Possible Players in Inflammatory Bowel Disease: Electrophysiological Evaluation of Their Role in Causing Functional Gastrointestinal Alterations in Murine Tissue

Rachele Garella *,†, Francesco Palmieri † and Roberta Squecco

Department of Experimental and Clinical Medicine, Section of Physiological Sciences, University of Florence, 50134 Florence, Italy; francesco.palmieri@unifi.it (F.P.); roberta.squecco@unifi.it (R.S.)

* Correspondence: rachele.garella@unifi.it

† These authors equally contributed to the manuscript.

Abstract: Inflammatory bowel disease (IBD) is a clinical condition of the gastrointestinal tract that has significant incidence in childhood. Major symptoms include abdominal pain, dyspepsia, delayed gastric emptying, anorexia, diarrhea and weight loss. IBD etiopathogenesis is multifactorial, with a proven involvement of cytokines. In this regard, cytokines like resistin and adiponectin produced by adipose tissue play a crucial role in inflammation. Particularly, resistin seems related to IBD severity and is considered a promising marker of disease occurrence and progression. Unraveling its mechanism of action and downstream effectors is mandatory when designing novel therapies. This preclinical study aims to further elucidate the action of resistin in causing functional gastrointestinal alterations, comparing it with the well-defined effect of adiponectin. To this end, we carried out electrophysiological analysis on murine gastric fundus. We found that resistin, similarly to adiponectin, increases smooth muscle cell (SMC) capacitance, indicative of cell surface remodeling, which is consistent with relaxation. However, contrary to adiponectin, resistin unalters membrane potential and inward Ca^{2+} entry and scarcely affects outward current, suggesting its inefficacy in markedly modifying electrical phenomena on the SMC membrane. This outcome, supporting the role of resistin in gastrointestinal distention, as observed in IBD, rules out a strikingly direct effect on SMCs.

Keywords: inflammatory bowel disease; adipokines; resistin; adiponectin; electrophysiology; proinflammatory cytokines; delayed gastric emptying; gastrointestinal motility; reduced appetite; weight loss

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1. Introduction

Inflammatory bowel disease (IBD) is a worldwide clinical condition with a significant incidence in childhood that is worthy of attention [1,2]. It is characterized by extended inflammation occurring throughout the whole digestive tract, namely the esophagus, stomach, and small and large intestine. IBD may also be associated with metabolic conditions and can often develop extraintestinal manifestations, with an impact on different organs, such as the liver. For instance, it has recently been shown that non-alcoholic fatty liver disease was observed in IBD patients, with a two times higher risk compared to healthy subjects [3].

The principal manifestations of IBD in pediatric patients are Crohn's disease and ulcerative colitis, with a frequent involvement of the upper gastrointestinal tract and with a highly associated presence of focally enhanced gastritis [4]. IBD is also accompanied by gastrointestinal distension [5], leading to a number of major symptoms that include abdominal pain, dyspepsia, nausea and vomiting, anorexia, constipation, diarrhea, and weight loss. A subset of subjects present with delayed gastric emptying [6]. Notably, when IBD occurs in children, it shows a more complex course and manifests in the form of a more substantial disease compared to IBD beginning in grown-ups, thus requiring aggressive therapeutic strategies [7,8] to promptly avoid incurring harmful effects on growth. Therefore, it would

be desirable to recognize the risk of incurring IBD in time and find possible strategies for IBD prevention, especially in childhood. In this regard, several studies have been focused on finding circulating molecules useful as early markers (pre-diagnosis metabolites) that are associated with the risk of IBD. Of interest, IBD has been found to be associated with prediagnostic perturbances in metabolic pathways. In particular, recent research recognized some specific metabolic pathways disturbed years before IBD diagnosis, involving the biosynthesis of steroid hormones, fatty acids, primary bile acids, and the metabolism of amino acids and nitrogen [9]. An in-depth elucidation of these metabolic pathways and of the related metabolites could unveil innovative biomarkers and provide new insights into the pathogenesis of the disease. The etiopathogenesis of IBD is, in fact, complex and multifactorial, involving biological/genetic and environmental factors, with stress playing a crucial role [10] and with the direct and indirect involvement of cytokines. In this regard, latter studies on IBD patients put forward the important role of adipocytes, which could take active part in systemic immune responses, even if a clear consensus is still lacking [11]. Adipose tissue, which has been classically considered an energy depot, has recently emerged as a true endocrine organ, producing peptide hormones and cytokines, namely adipokines, and plays a crucial role in inflammatory processes. Notably, alterations in adipokine profiles have been observed in several digestive diseases and disorders of the gastrointestinal tract, such as hepatic, biliary tract, pancreatic, and gastrointestinal diseases, suggesting that adipokines could potentially contribute to the identification of prognostic biomarkers for this correlated digestive condition. Currently, the role of adipokines in gastrointestinal disorders remains to be clarified, together with the fact of whether an altered adipokine profile is a consequence of the disease or promotes disease progression [12]. Some of these adipokines, such as leptin, resistin (RES), and adiponectin, seem to be altered in IBD patients and are related to disease severity [11]. Remarkably, IBD patients usually show lower levels of leptin and higher levels of adiponectin and RES in their blood [13]. In particular, RES is a cysteine-rich adipokine produced in humans by adipocytes as well as by immune cells and macrophages and can be considered a promising inflammatory marker due to the significant connections between adipose tissues and inflammatory response. RES reveals a proinflammatory profile in a number of different clinical conditions [14], and, as stated above, its serum levels are increased in IBD patients [15]. Accordingly, to assess the true validity of RES as a key player in disease occurrence and progression, growing interest is arising in clarifying the precise role of its molecular isoforms, the mechanisms of action and signaling pathways, the downstream effectors, and the major targets as a starting point to guide novel therapeutic interventions.

In order to consider RES and other adipokines as molecules with actual applications from a translational perspective in IBD, this study aimed to shed light on their mechanism of action. Precisely, we intended to clarify the molecular targets of RES, testing its effect on gastric fundus smooth muscle cells, as previously assessed for adiponectin [16]. In particular, we focused on the gastric smooth muscle, taking advantage of our previous studies on adipokines that demonstrated their ability to favor gastric smooth muscle layer relaxation [16], affecting mechanical response [17]. The present electrophysiological results suggest that, contrary to adiponectin, RES does not seem to cause a significant direct effect on smooth muscle cell (SMC) excitability since it does not appreciably affect ionic membrane channels. While supporting earlier findings indicative of the modulatory action of this adipokine mainly directed on nitrergic neurotransmission [17], we have added a new piece of knowledge on the mechanism of action of RES, proving its role in gastrointestinal distention that is ascribable, at the moment, only to NOS activation [17]. Although this study offers a stimulating background to the possibilities of using RES and/or its targets and intermediates of the signaling pathway as diagnostic or prognostic markers or even novel therapeutics to face and defeat IBD, its precise mechanism of action and the identification of downstream effectors need further elucidation.

2. Results

2.1. RES Slightly Affects Gastric Smooth Muscle Cell Membrane Passive Properties in Mice

To elucidate and extend the functional role of RES on gastric wall relaxation that goes beyond its proinflammatory role, we investigated its possible direct effects on gastric fundus SMC excitability. We first focused our attention on resting membrane potential (RMP) analysis. As previously described in detail [16], electrophysiological recordings were obtained using the microelectrode technique on murine gastric fundus tissue strips. RMP was measured before (CTRL) and after the addition of RES (100 ng/mL) to the bath medium. We chose this hormone concentration because it was already proven by our research group to be effective in affecting gastric motor response in the same type of preparation [17]. According to the procedure previously used for other adipokines [16], recordings were taken 15 min after the administration of the hormone to the external solution. RES addition usually caused no alterations in terms of RMP or, at least, slight hyperpolarization of 1–2 mV. However, the analysis of the RMP mean values showed no significant differences between the two experimental conditions (Figure 1 and Table 1), indicating that RES, contrary to the previous findings on adiponectin [18], is not able to appreciably modify the RMP, and thus, SMC excitability.

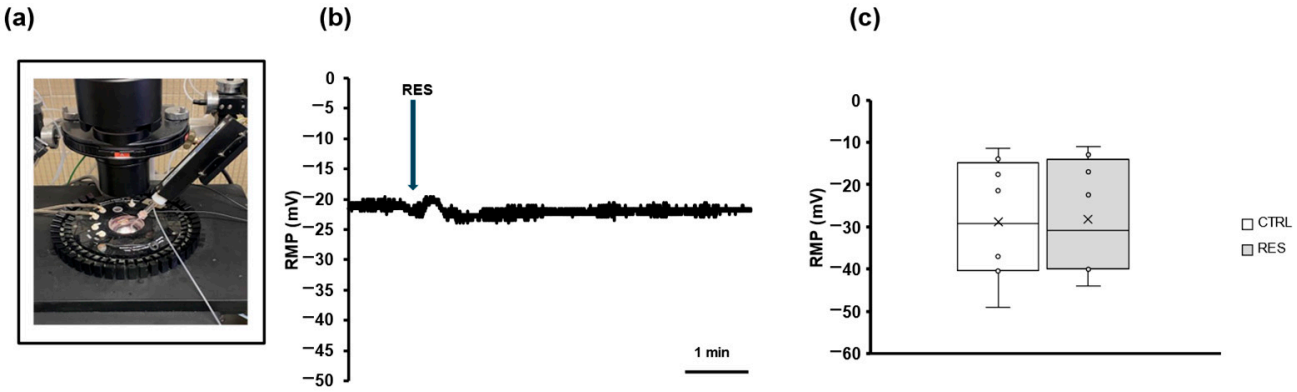

Figure 1. Effect of RES on SMC resting membrane potential (RMP). (**a**) Detail of the experimental recording chamber. (**b**) Representative example of RMP tracings (in mV) evoked from a gastric fundus SMC before and after the addition of RES (100 ng/mL), indicated by the arrow; (**c**) RMP values (in mV) from untreated cells (CTRL, $n = 8$) and after RES addition (RES, $n = 8$). Data values represented as mean ± SD are listed in Table 1. $p > 0.05$ vs. CTRL (Student's t test, $p = 0.41$).

Table 1. Effect of RES on passive properties of gastric fundus smooth muscle cell (SMC) membrane.

	CTRL	RES
RMP (mV)	−28.87 ± 14.32 $n = 8$	−28.25 ± 13.7 $n = 8$
Cm (pF)	24.54 ± 2.7 $n = 7$	39.77 ± 16.78 * $n = 7$
Gm (nS)	10.06 ± 6.41 $n = 14$	15.91 ± 9.23 $n = 17$

Data are expressed as mean ± SD. n = number of investigated SMCs, * $p \leq 0.05$ vs. CTRL (Student's t test).

To further investigate the possible action of RES on the bioelectrical features of SMCs, we analyzed the effect of this adipokine on the membrane's passive properties. In particular, using the voltage–clamp pulse protocol shown in Figure 2a, we recorded current traces that allowed us to estimate the value of cell capacitance (Cm, Figure 2b) as an index of cell surface extension and/or morphology and membrane conductance (Gm, Figure 2c) as an index of membrane permeability. The addition of RES to the bath solution, similar to what was previously observed after adiponectin addition [19], significantly increased membrane capacitance ($p = 0.050$, Student's t test), likely suggesting that RES was also able to induce an equivalent change in membrane morphology and cell shape. However, in contrast to adiponectin, it did not induce significant changes in membrane conductance ($p = 0.10$, Student's t test), probably indicating a negligible action on basal ion channel opening.

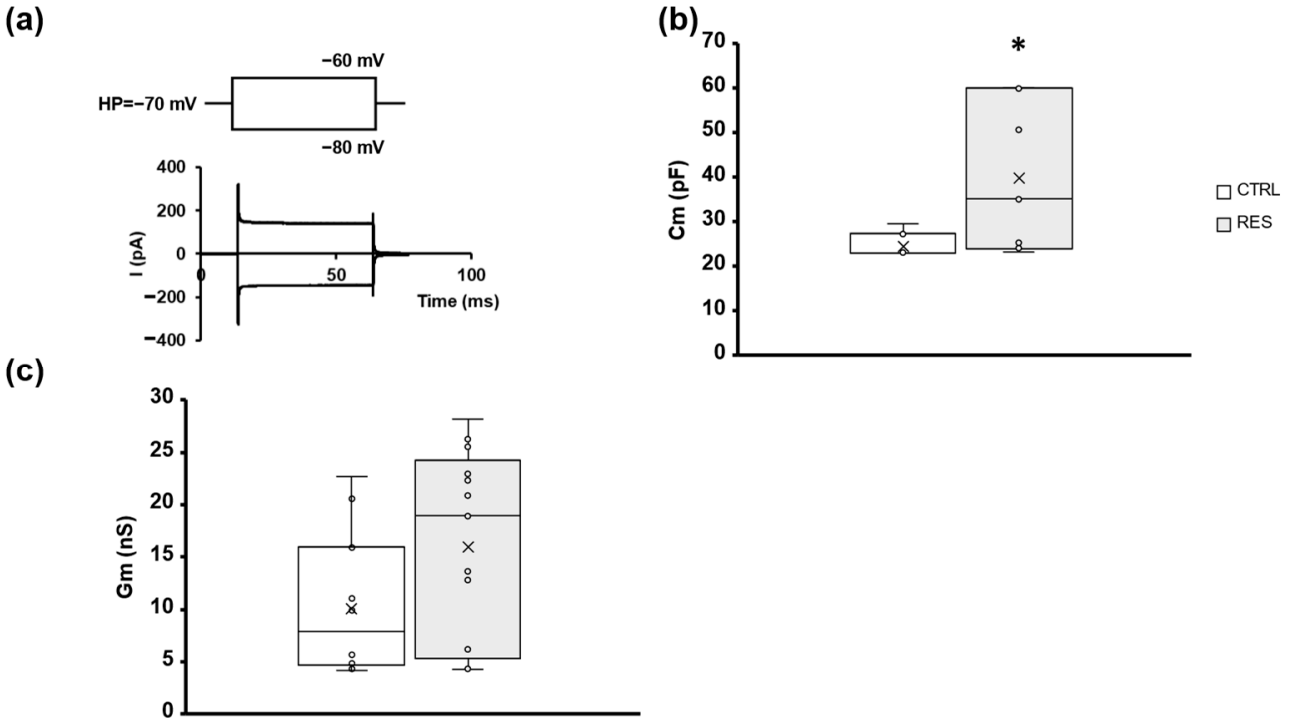

Figure 2. Effect of RES (100 ng/mL) on SMC membrane passive properties: (**a**) voltage–clamp protocol used for evaluation of membrane's passive properties, consisting of a ±10 mV voltage pulse, applied from a holding potential (HP) = −70 mV (top) and representative current response (pA) (bottom); (**b**) membrane capacitance, Cm (pF); (**c**) membrane conductance, Gm (nS). * $p < 0.05$ vs. CTRL (Student's t test). Parameter values as mean ± SD are listed in Table 1.

2.2. Effects of RES on SMCs Ion Currents

To investigate a further functional aspect of RES action, we evaluated its possible effect on gastric fundus SMC ion currents, which are mainly involved in SMC excitability and contractility, namely outward K^+ and inward Ca^{2+} currents, respectively. Accordingly, current responses were evoked using a suitable voltage–clamp step pulse protocol (shown in Figures 3 and 4, inset) before and after the addition of RES to the bath solution.

Table 2. Analysis of the ion current steady-state activation curves in gastric fundus SMCs in the absence and presence of RES.

	Outward Current		Inward Current	
	CTRL	**RES**	**CTRL**	**RES**
$V_{0.5}$ (mV)	4.50 ± 23.01 n = 3	18.04 ± 7.96 n = 3	3.09 ± 7.17 n = 3	0.00 ± 0.21 n = 3
k (mV)	20.00 ± 4.61 n = 3	14.55 ± 3.84 n = 3	17.24 ± 2.04 n = 3	20.47 ± 3.45 n = 3
R^2	0.991	0.988	0.994	0.992

Boltzmann function $G/Gmax = 1 - 1/\{1 + e[(V_m - V_{0.5})/k]\}$; Boltzmann function parameters: G/Gmax = conductance normalized to its maximal value; V_m = membrane potential; k = slope factor; $V_{0.5}$ = half-maximal voltage value; R^2 = coefficient of determination; n = number of cells.

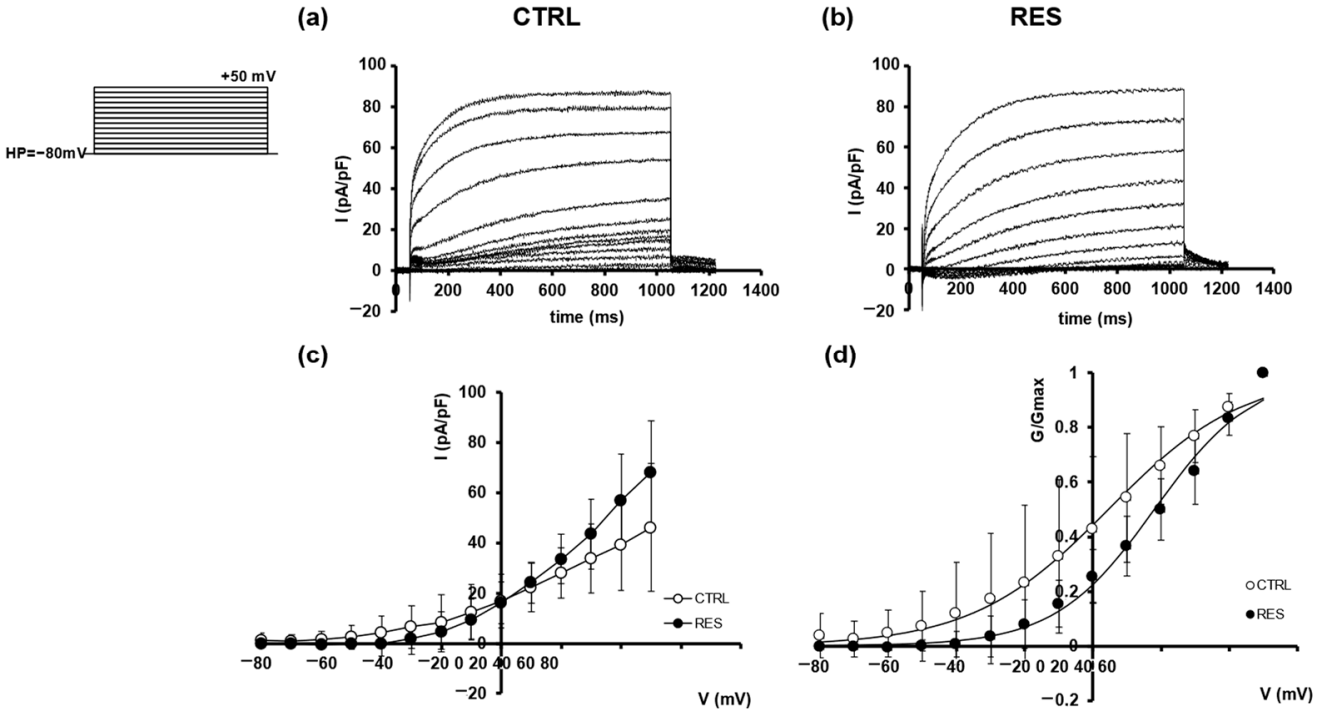

Figure 3. Effects of RES on outward K^+ currents evoked from murine gastric fundus SMCs. Representative tracings of outward ion currents (in pA/pF) (voltage–clamp step pulse protocol in the inset) recorded from a gastric fundus SMC before (**a**) and after the addition of RES (100 ng/mL) (**b**) to the bath medium. (**c**) I-V plot (in pA/pF) reporting the mean outward currents related to all the experiments carried out as a function of the voltage step applied (CTRL: open circles; RES: filled circles). (**d**) Normalized conductances fitted with the Boltzmann function: $G/Gmax = 1 - 1/\{1 + e[(V_m - V_{0.5})/k]\}$ (superimposed smooth lines). Boltzmann parameters are listed in Table 2. Data are expressed as mean ± SD (n = 3). $p > 0.05$ vs. CTRL (Student's t test). The current traces displayed in (**a**,**b**) are recorded from the same SMC.

The addition of RES was not able to significantly change the amplitude of the outward currents compared to the CTRL (Figure 3a,b), in accordance with the slight, not significant alteration of the RMP observed. The I-V plot analysis of all the experiments carried out (Figure 3c) confirmed that the differences between the currents measured at the steady state in the two conditions were not statistically significant, even if the mean current recorded in the presence of RES tended to be smaller for negative voltage steps and bigger for positive ones. We then calculated the normalized average conductances (G/Gmax) (steady-state activation curves) [20] of ion currents, as described in the Materials and Methods

section. These data are reported in Figure 3d to show the dependence of G/Gmax on the voltage test pulse and are fitted with a least-squares criterion procedure with a Boltzmann function (see Materials and Methods). In both conditions, we observed a voltage-dependent increase in conductance: in the control condition, G/Gmax increased with the membrane depolarization for voltage test pulses positive to −60 mV. In contrast, in the presence of RES, G/Gmax seemed to increase for voltages positive to −30 mV, resembling the typical behavior of delayed rectifier K^+ channels [21]. The Boltzmann function fitted to G/Gmax data showed about a 15 mV positive shift of the half-maximal voltage value, $V_{0.5}$, in the presence of RES (Table 2), suggesting that K^+ channels may vary their voltage dependence of activation. We may tentatively propose that the presence of RES in the bath solution inhibits the activation of a low-threshold K^+ current observed in the CTRL condition, having properties similar to $K_{A,}$ for instance [22].

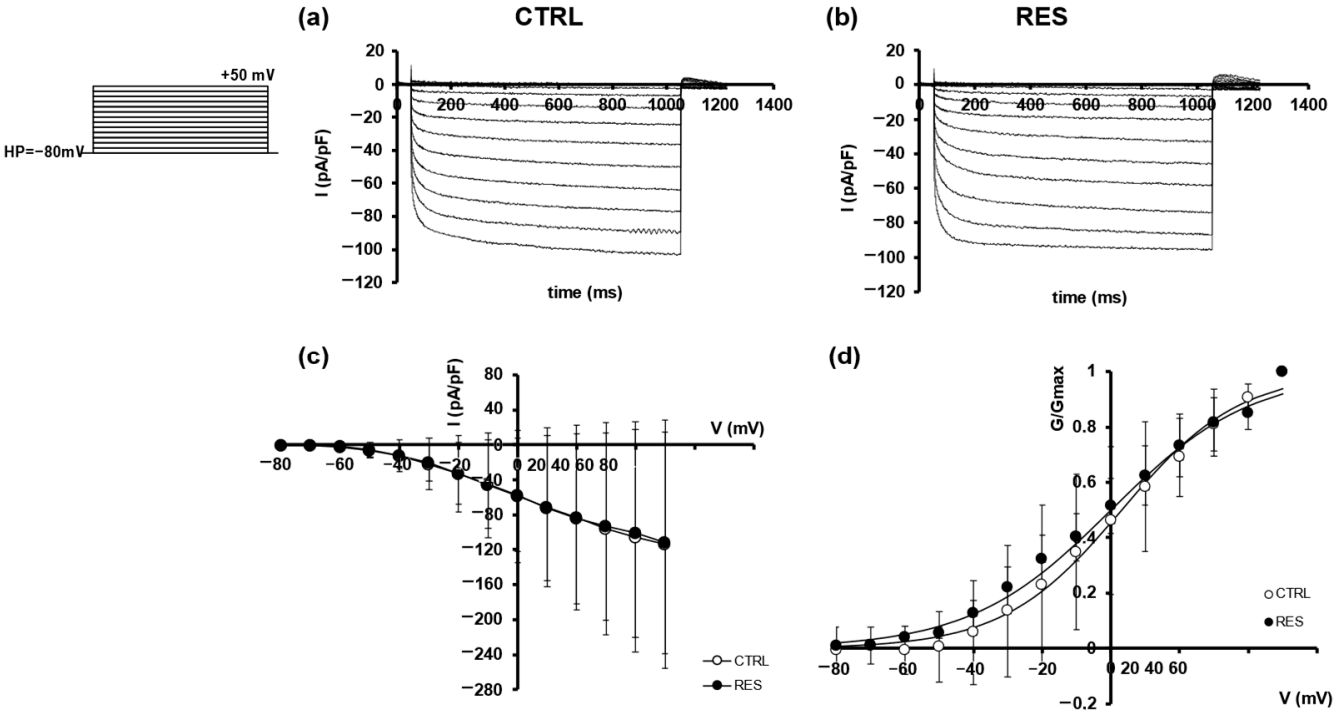

Figure 4. Effects of RES on inward Ca^{2+} currents evoked from murine gastric fundus SMCs. Representative tracings of inward ion currents (in pA/pF) (pulse protocol in the inset) recorded from a gastric fundus SMC before (**a**) and after the addition of RES (**b**) to the bath medium. (**c**) I-V plot (in pA/pF) reporting the mean inward ion currents related to all the experiments done as a function of the voltage step applied (CTRL: open circles; RES: filled circles). (**d**) Normalized conductances fitted with the Boltzmann function: $G/Gmax = 1 - 1/\{1 + e[(V_m - V_{0.5})/k]\}$ (superimposed smooth lines). Boltzmann parameters are listed in Table 2. Data are expressed as mean ± SD ($n = 3$). $p > 0.05$ vs. CTRL (Student's *t* test). The current traces displayed in (**a**,**b**) are recorded from the same SMC.

Similarly, the addition of RES did not alter the size of the inward Ca^{2+} current (Figure 4a,b); no statistically significant differences were observed between the currents recorded in the control condition and after the addition of RES. Again, the I-V relation confirmed this behavior (Figure 4c), and G/Gmax analysis showed two almost overlapping curves for CTRL and RES (Figure 4d). This result indicates that RES barely alters inward Ca^{2+} influx, which can be useful for SMC contraction, contrary to what was observed with adiponectin, which consistently caused a reduction in inward Ca^{2+} current [16]. Thus, we suggest that RES may affect SMC relaxation via a mechanism that does not depend on electromechanical coupling alterations but rather involves Ca^{2+}-independent pathways [23].

3. Discussion

IBD is a multifactorial clinical condition of the gastrointestinal tract that is typically characterized by chronic and relapsing inflammation. In recent years, novel drugs and therapies have ameliorated the percentage and degree of remission in IBD patients, targeting multiple mechanisms underpinning the disease and often leading to complete or satisfactory mucosal healing [24]. However, a number of patients with IBD can show refractory gastrointestinal symptoms, even in the absence of objective inflammation [25], supporting the idea of a not totally stringent correlation between mucosal inflammation and gastrointestinal symptoms [26]. Furthermore, among these symptoms, a subset of patients display delayed gastric emptying, likely ascribable to an altered release of gastrointestinal hormones like GLP-1, which can reinforce gastric emptying disturbances [6]. These observations push a search for "functional" gastrointestinal conditions that could occur as a consequence of inflammation and of possible molecules with an effective action on them. In this regard, a deeper understanding of gastrointestinal physiology and, eventually, of gut–brain communication, may have clinical relevance and lead to valid therapeutic targets for the treatment of several conditions [27].

3.1. Adipokines and IBD

Regarding potential molecules with a role both in gastrointestinal activity and inflammatory processes, there is a rising interest in adipokines such as leptin, visfatin, adiponectin, and RES [11,12]. In particular, adiponectin is an anti-inflammatory hormone [28], triggering almost well-defined signaling pathways, and can thus have a pivotal role in the gravity of IBD and/or therapy. In contrast, RES has clear proinflammatory properties [14] and plasma level alterations have indeed proven to have a key role in the pathogenesis of IBD [29] and other clinical conditions. As well as being related to IBD severity, RES is commonly associated with glucose homeostasis and body composition, sharing these features with other adipokines like adiponectin. Therefore, RES can be considered as a link between inflammatory responses and the adipose depot. Comparably to adiponectin, RES has shown another action in the context of the gut–brain axis, that is, the ability to affect gastric fundus tone [17]. This modulatory action, which facilitates gastric wall relaxation, could contribute to the delay in gastric emptying observed in IBD [6]. The above-mentioned effect could be achieved via the activity of nitrergic transmission, as confirmed by the increased expression of neuronal nitric oxide synthase (nNOS) observed in the myenteric plexus after RES exposure [17]. As a result, it is expected that RES could enhance NO release from inhibitory enteric motor neurons; however, to our knowledge, the precise molecular mechanism of action has not been defined yet.

3.2. Possible Mechanisms of Action of RES

It has been steadily accepted that the release of NO from inhibitory enteric motor neurons usually allows NO-mediated SMC relaxation, activating via the soluble isoform of guanylate cyclase (sGC) in target cells and producing cyclic guanosine monophosphate (cGMP) as a second messenger. In this regard, a possible direct effect of RES at the muscular level, as observed for adiponectin, in addition to its activity on nitrergic neurotransmission, has not been investigated through the use of electrophysiological analysis up to now. A previous study [17] put forward that RES might reinforce the action of NO action at the muscular level through the sGC/cGMP pathway, as described for adiponectin [16]. In this study, we actually intended to consider this hypothesis: if this was the case, we should have seen an effect on SMCs similar to that reported for adiponectin, with consistent alterations of membrane bioelectrical features, such as RMP hyperpolarization, a substantial reduction in inward current, and K^+ current enhancement. In contrast, we found that RES treatment caused only negligible modifications to the SMC RMP, membrane conductance, and ion current amplitudes, instead suggesting the involvement of sGC/cGMP-independent NO signaling pathways. Based on the previous literature, we can speculate the possible participation of different NOS/NO-related processes, such as S-nitrosation and the oxidative

addition of NO to cysteine residues of several proteins, representing a mechanism for the redox-based physiological regulation of a wide range of critical functions [30]. Additionally, the catalytic function of NOS to generate superoxide radicals, as observed in the central nervous system [31], is worthy of consideration. This NOS activity may, in fact, generate the superoxide that is reported to actually inhibit some K^+ channels [32].

Accordingly, in our gastric preparation, we observed that even if RES induced only a slight and not statistically significant K^+ current alteration, this was partly opposite to what was observed after adiponectin treatment [18] since RES seemed to reduce the K^+ current, at least for negative voltage steps. Notably, as IBD is one of the numerous diseases commonly associated with prolonged or persistent exposure to non-physiological levels of reactive oxygen species [33], the involvement of reactive oxygen and, possibly, nitrogen species could be a very interesting option to be considered as possible effectors of RES action in mediating SMC changes in mechanical response. In this regard, the previous literature reported that excessive reactive oxygen species play a significant role in disrupting cytoskeleton integrity [34], and this may inexorably lead to a change in cell shape and contractile machinery. In addition, although IBD has long been associated with oxidative injury, substances with "antioxidant" activity reveal contrasting results in clinical trials focusing on IBD [35]. The hypothesis that NOS also generates reactive oxygen and nitrogen species deserves further investigation in future experiments, both in murine models and possibly replication on human gastric biopsies. If experiments planned for human samples confirm the data obtained relating to murine gastric fundus, we can reasonably suppose that, in addition to the proven proinflammatory action, RES could contribute to worsening some IBD symptoms by increasing NOS activity and decreasing gastric tone, delaying gastric emptying and increasing GI distention. In that case, RES activity may resemble the already advanced action of gastrointestinal hormones like GLP-1 in reinforcing gastric emptying disturbances [6].

3.3. Significance of RES Functional Effect on Gastric Fundus in IBD

Interestingly, these pro-relaxing effects on the gastric fundus could reinforce the peripheral satiety signals, as proposed for adiponectin in murine models [36], through the vagal afferents from the stomach directed to the *nucleus tractus solitarius* (NTS), thus reducing appetite, worsening anorexia and, consequently, malnutrition and weight loss. These implications for IBD have a crucial influence on children since they can also have detrimental repercussions on growth and development [8].

3.4. Limitations of the Study

Although this research follows a well-consolidated procedure to evaluate the effect of adipokines and their mechanism of action, as reported in previously published studies [16–18], this preclinical study was only conducted on ex vivo gastric fundus wall obtained from a mouse model. Mice are usually assessed as valid and standardized models to investigate basic biological processes [16]; however, using human gastric tissue from biopsies or surgical samples would be more reliable. Nonetheless, human samples are quite hard to obtain in amounts large enough (especially from healthy donors) to perform a powerful statistical analysis since this depends on subject/patient availability. In any case, we are planning to move in that direction. Another limitation of our experimental design was the use of samples from only female mice. This choice was primarily taken to be in line and comparable with our previous experiments on adipokines [16–18], even if this may limit the generalizability of the results to male mice. To overcome these limitations, further studies could certainly be performed on male animals. Additionally, the choice of using intentionally healthy animals to perform our experiments might seem limiting. However, this initial step is necessary to test the effects of RES and elucidate their mechanisms of action on the gastric fundus under physiological conditions to unravel the precise role of RES on healthy tissue and to consider rising levels of RES as the cause of alterations to gastric function. For the future development of the study, the effects of RES could be

tested on inflammatory tissue and, finally, the use of IBD animal models could be useful in undertaking in vivo studies. The use of further rodent models, as well as of different tracts of the gastrointestinal apparatus, could be valid options to further confirm these data.

3.5. Novel Findings

To sum up, our preliminary results on murine gastric fundus indicate that RES is able to induce increased SMC membrane capacitance, as previously observed with adiponectin [16], indicating its ability to alter membrane morphology and cell shape, likely related to a more relaxed status. However, contrary to adiponectin, RES does not significantly modify electrical phenomena occurring on the SMC membrane and does not substantially affect electromechanical coupling: it does not seem to alter excitability, it scarcely changes outward current and does not affect inward calcium entry through membrane ion channels. In this regard, we can deduce that RES facilitates SMC relaxation via a mechanism that involves Ca^{2+}-independent pathways, such as Rho-associated protein kinase (ROCK) [23]. ROCK has been reported to play key roles in cytoskeletal assembly [37,38], whereas ROCK inhibitors have been presumed to decrease actomyosin contraction [39], leading to major cell relaxation. Indeed, the integrin/ROCK pathway has proven to be involved in oxidative injury by regulating cytoskeleton remodeling [40]. The above observations argue in favor of the absence of a considerable number of proteins/receptors at the SMC membrane level that are able to respond directly to RES or to its signaling paths that would lead to adjustments in membrane properties. As a consequence, if this hypothesis were confirmed in further studies, we would have a smaller number of possible pharmacological targets on the gastrointestinal wall that are available to counteract IBD.

3.6. Concluding Remarks

Although negative, this outcome adds a piece of knowledge to the complex mechanism of action of RES, supporting the role of this hormone in gastrointestinal distention, as observed in IBD, attributable at the moment only to neuronal NOS activation and NO production [41], without a direct effect on SMCs. The final downstream effectors have yet to be clarified: further investigations could be directed to test the role of reactive oxygen and nitrogen species and cGMP-independent NO signaling pathways triggered by RES. Experiments should be carried out on rodents and then replicated in human samples, since basic research studies are necessary in pushing the progress of novel therapeutics in the early fight against IBD. In any case, further research is generally needed to confirm any potential clinical relevance of RES and adipokines in IBD management.

4. Materials and Methods

4.1. Animals and Treatments

In accordance with our previously published papers [16,18], C57BL/6J (Charles River, Lecco, Italy) 8/12 weeks female mice were used for our experiments, fed with standard laboratory food and water and subjected to a photoperiod of 12 h of light and 12 h of dark at a controlled temperature (21 °C). The animals were rapidly sacrificed via cervical dislocation to minimize suffering (at least 5 mice were used). After the removal of the stomach from the abdomen, we isolated the gastric fundus and opened it along the small curvature; then, we cut 2 or 3 full-thickness (2 × 10 mm) longitudinal strips from this region. Each strip was first tested without any treatment (CTRL) and then tested again at least 15 min after the addition of RES (100 ng/mL) to the bath medium. The concentrations are given as the final concentration in the bath. The experimental solutions were made before conducting the experiment. RES stock solution was kept at −20 °C. Recombinant mouse RES, as well as all the drugs and reagents used, were obtained from Sigma Chemical (St. Louis, MO, USA).

4.2. Tissue Sample Preparation and Electrophysiological Records

For electrophysiological recordings using the microelectrode technique, each excised stomach was cleaned with Krebs–Henseleit solution (KH) [16] consisting of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, and 2.5 mM $CaCl_2$ (pH 7.4 with NaOH). First, we accurately removed the mucosa and submucosa under a dissecting microscope (Nikon, SMZ-1; Tokyo, Japan). The remaining tissue was fixed with the serous part upwards, and the connective tissue was eliminated in order to expose the smooth muscle layer. The sample obtained was finally fixed to the recording chamber with the serous part facing upwards (Sylgard-coated p35 Petri dish). During the experiments, the pinned tissue was continuously perfused with KH solution at a rate of 1.8 mL/min. A conventional high-resistance glass electrode was introduced into a smooth muscle cell of the longitudinal muscle layer for intracellular recordings [42]. A vertical puller (Narishige, Tokyo, Japan) was used to produce the microelectrodes from borosilicate glass tubes (GC150-7.5, Harvard Apparatus LTD, Holliston, MA, USA). The microelectrodes were usually filled with the following internal solution (mM): KCl 130, NaH_2PO_4 10, $CaCl_2$ 0.2, ethylene-bis (oxyethylenenitrilo) tetraacetic acid (EGTA) 1, MgATP 5, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/KOH 10 (pH 7.2 with tetraethylammonium hydroxide). Once filled, the resistance of the pipette was 60–70 MΩ. The patch pipette was located in a CV203BU head-stage (Axon Instruments, Foster City, CA, USA) that was connected to a three-way coarse micro-manipulator (Narishige, Tokyo, Japan) and to an Axopatch 200 B amplifier (Axon Instruments, Foster City, CA, USA), as detailed in earlier papers [43]. Stimulation protocols were run using the A/D-D/A interfaces (Digidata 1200; Axon Instruments) and Pclamp 6 software version 6.0.4 (Axon Instruments Foster City, CA, USA). In the current–clamp mode, we recorded the RMP of the SMCs using a pulse waveform of I = 0 pA before and after drug addition. We evaluated the passive properties of the SMC membrane (Gm and Cm) under voltage–clamp conditions by applying two 75 ms voltage steps, starting from a holding potential (HP) of −70 mV ranging from −80 to −60 mV. The activation of voltage-dependent ion channels was evoked in the SMCs held at HP = −80 mV by applying 1 s long voltage steps ranging from −70 to 50 mV in 10 mV increments. An interval of 20 s was given between one episode and the following to allow for recovery. Capacitive, linear leakage, and voltage-independent currents were canceled directly online using the P/4 procedure.

4.3. Data Analysis

Ionic currents were estimated and analyzed using Clampfit version 9.0 software (Axon Instruments). For a correct comparison of the currents arising from cells with different areas, the current size was normalized to the linear cell capacitance, Cm, that is, related to the cell surface area, and being the specific membrane capacitance value = 1 $\mu F/cm^2$. The current–voltage relation (I–V plot) was obtained by calculating the mean value of the current amplitudes measured at the end of the step pulse and normalizing them for cell capacitance and then plotting these data as a function of each voltage step applied. K^+ conductance was calculated using the function $G = I/(V - E_K)$, where G is the conductance for K^+, I is the K^+ current, V is the command voltage, and E_K is the equilibrium potential for K^+ determined by the extracellular and intracellular K^+ concentrations. Any G value was normalized for Gmax to obtain G/Gmax. The same procedure was applied for Ca^{2+} conductance. The normalized conductances were fitted to a least squares criterion procedure with the Boltzmann function: $G/Gmax = 1 - 1/\{1 + e[(V_m - V_{0.5})/k]\}$, where G/Gmax is the conductance normalized to its maximal value, V_m is the membrane potential, *k* represents the slope factor, and $V_{0.5}$ is the half-maximal voltage value. R^2 is the coefficient of determination that is a measure of the goodness of fit.

4.4. Statistical Analysis

The mathematical and statistical analysis of the data obtained from the experiments and graphs generation were executed using Microsoft Excel 2016(Microsoft, Washington,

DC, USA). To calculate if the data were normally distributed, we used an online Shapiro–Wilk test (https://www.statskingdom.com/shapiro-wilk-test-calculator.html; URL accessed on 5 march 2024). Statistical significance between two data sets was determined using Student's t test for paired data. n is the number of SMCs. All of the results are presented as mean $\pm$ SD. $p \leq 0.05$ represents statistical significance.

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