ASTERAND PRELIMINARY PROJECT REPORT 1.0

PROJECT TITLE **Assessment of AQIX® RS-I solution in functional pharmacology experiments using human isolated tissue**

PROJECT CODE **AST / PZ / 1724**

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1. INTRODUCTION

Aqix Ltd (formerly Res-Del International Ltd) would like to demonstrate the advantages of using their AQIX® RS-I solution for transportation of human organs and tissue biopsies and their subsequent use in functional pharmacology experiments utilising the same, AQIX® RS-I solution also for the normothermic perfusion of isolated organ and tissue biopsies.

Using human colon longitudinal smooth muscle, obtained from a single donor (Donor/ EXP32226), the effects of carbachol and electrical field stimulation (EFS) have been tested in a comparative study on the effects of AQIX® RS-I vs. Krebs' solution.

1 MATERIALS AND METHODS

1.1 TISSUE SAMPLES

Macroscopical human colon biopsies with no clinical or pathological indicators of any disease were utilized in this trial.

1.2 STANDARD CONDITIONS

a) Samples of human colon biopsies (Donor/EXP32226) were cut into half upon retrieval. One half was placed into a sample polypropylene pot containing AQIX® RS-I and the other half into a pot containing phosphate buffered saline (PBS). The pots containing the samples were transported to a laboratory for functional analyses on wet ice (0-4°C).

b) Upon receipt of the samples at the laboratory, the AQIX® RS-I stored colon samples were placed in freshly, carbogenated AQIX® RS-I, whereas the PBS bathed colon were placed in freshly, carbogenated Krebs' physiological salt solution. The composition of the Krebs' solution was (mM): NaCl (118.1), KCl (4.69), MgSO4.7H2O (1.18), KH2PO4 (1.18), D-glucose (11.1), NaHCO3 (25.0) and CaCl2.6H2O (2.5).

c) The mucosa was removed from all colon samples and intertaenial sections of the smooth muscle was cut into 4 longitudinal strips (referred to as 'Tissues') ~10mm long and ~3mm wide.

d) Tissues were then suspended in 10ml tissue baths, gassed with 95% O2/5% CO2 and maintained at a temperature of 37°C. The baths contained AQIX® RS-I for those Tissues transported and dissected in AQIX® RS-I, whereas the baths contained Krebs' solution for those Tissues transported in PBS and dissected in Krebs' solution.

Isometric tension was measured for each Tissue.

Tissues were then set under an initial resting tension of 15mN.

1.3 OUTLINE PROTOCOL

Tissues were allowed to equilibrate for a period of 60 minutes, during which time they were washed 3 times and re-tensioned to 15mN if required.

Following equilibration, concentration-effect curves (CEC) to the muscarinic agonist, carbachol ($1x10^{-9}$M to a concentration causing a maximal effect, log unit increments), were constructed on all Tissue samples.

Tissues were washed, re-tensioned and allowed to recover for ~1 hour. Following recovery, the Tissues underwent electrical field stimulation (EFS). Frequency-response curves were constructed on all Tissues using the following parameters: 0.1, 0.5, 1, 5, 10, 20 and 30Hz at 15v with a 1ms pulse width for 10s every 60s.

Tissues were then washed, re-tensioned and allowed to recover for ~1 hour. Following recovery, the Tissues were then stimulated with a submaximal frequency from the EFS curve and then continuously stimulated for a period of 2 hours to assess the stability of the EFS-evoked contractions.
Following the stimulation period, any remaining EFS-induced contractions were challenged with tetrodotoxin (TTX, $1x10^{-6}$M) to confirm EFS-induced nerve stimulation.

1.4 DATA ANALYSIS

1. Responses to carbachol have been measured as mean change in baseline in both AQIX RS-I and Krebs' tissues and, in the AQIX RS-I tissues only, mean response of spontaneous activity and mean maximum response of spontaneous activity. pEC_{50} values have been calculated from mean change in baseline in the Krebs' tissues and mean response of spontaneous activity in the AQIX RS-I tissues.

2. Responses to EFS frequency-response curve (FRC) are measured as mean mN response at each frequency.

3. Responses over the 2-hour EFS period have been measured as percentage change of initial EFS-evoked contractions.

4. Responses to TTX have been measured as percentage change of pre-TTX EFS-evoked contractions.

2. RESULTS

The data obtained from Donor/EXP32226 biopsied colon are summarised below in Table 1 and Figures 1 to 7.

2.1 Carbachol concentration-effect curve (CEC)

In the Krebs' immersed tissues, carbachol caused small, concentration-dependant tonic contractions of basal tone in all four tissues. In contrast, in the AQIX RS-I immersed tissues, carbachol caused large concentration-dependant, continuous, phasic contractions in all four tissues tested (see, Figs.1 & 7).

2.2 EFS FRC

EFS FRC contractions were more pronounced in the AQIX RS-I immersed tissues, with all four tissues responding, than in the Krebs' immersed tissues, where only one tissue showed a clear response (see, Fig.2). However, neither AQIX RS-I or Krebs' tissues showed clear, frequency-dependant contractions during the FRC (see Figs. 4 & 5).

2.3 EFS sub-maximal response and treatment period

EFS, at sub-maximal frequency (20 Hz), caused clear contractions in all tissues, however, in three of the Krebs' immersed tissues, these were very small (see, Fig.3). Over the 2-hour EFS period, there was a clear increase in the EFS-evoked contractions in two of the AQIX RS-I immersed tissues (Figs. 3 & 6;Tissues 5 and 7), whereas the Krebs' immersed tissues showed no clear change, including in the one clear responsive tissue (Tissue 3), where EFS-evoked contractions were well maintained throughout the treatment period.

2.2 Effect of TTX on EFS-evoked contractions

In the Krebs' tissues, TTX caused a transient inhibition of EFS-evoked contractions in the one clear responding tissue (Fig.3;Tissue 3) but had no clear effect on the small contractions in the other three tissues. In the AQIX RS-I tissues, TTX caused a clear inhibition of EFS-evoked contractions in two of the four tissues, these were the same two tissues that exhibited an increase in contractions over the 2-hour time period (Fig.3;Tissues 5 and 7), whilst causing a clear potentiation of contractions in the other two tissues (Fig.3;Tissues 6 and 8).

Table 1. **Summary of the contractions to carbachol and electrical field stimulation (EFS) in human isolated colon, longitudinal smooth muscle (Donor/EXP32226)**

Tissue	Carbachol CEC					EFS FRC	EFS sub max			TTX ($1x10^{-6}$M)	
	pEC_{50}*	slope*	max[a] (mN)	max[b] (mN)	max[c] (mN)	max (mN)	Frequency	mN	% EFS response remaining (at 120mins)	mN	% change EFS response
K&H-1	5.9	1.2	12	12	12	0.4	20Hz	0.4	63	0.2	66
K&H-2	6.0	1.1	9	9	9	0.4	20Hz	0.2	129	0.0	-3
K&H-3	6.4	1.0	32	32	32	10.1	20Hz	13.0	103	-9.1[d]	-68[d]
K&H-4	6.2	1.2	13	13	13	0.7	20Hz	0.5	95	-0.2	-35
AQIX-5	6.4	2.4	95	45	319	4.8	20Hz	1.1	814	-7.3	-84
AQIX-6	6.4	2.4	41	22	79	3.9	20Hz	1.2	150	5.4	288
AQIX-7	6.7	7.8	42	21	181	15.9	20Hz	7.9	166	-10.0	-77
AQIX-8	5.9	5.4	51	44	213	6.9	20Hz	1.9	70	9.0	696

CEC = concentration-effect curve
NC = not calculable
NA = not applicable
* = calculated from max[a] values
[a] = mean max response of spontaneous activity
[b] = baseline
[c] = maximum response
[d] = transient inhibition

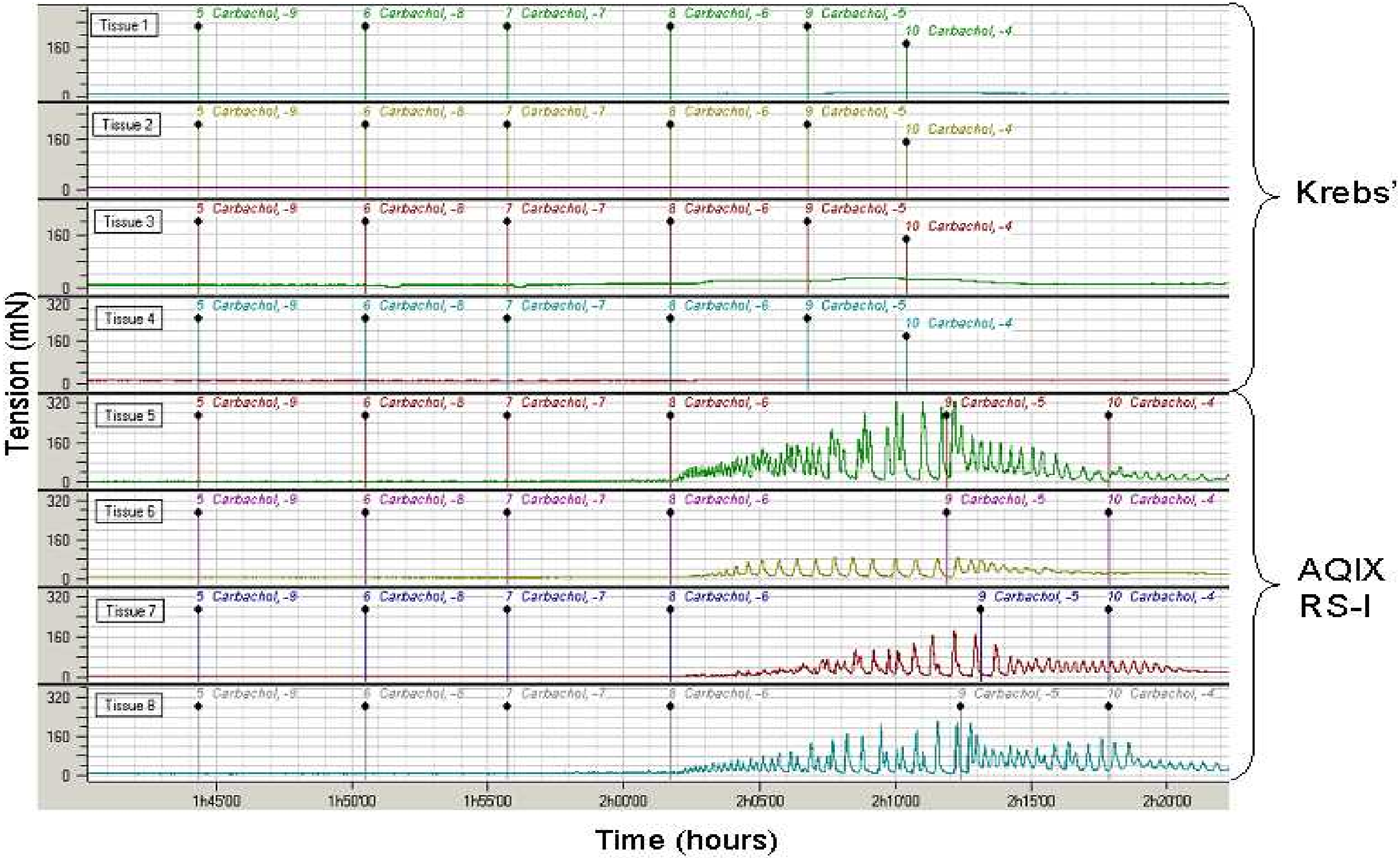


Figure 1. Experimental traces showing effects of carbachol on basal tone in isolated Human colon, longitudinal smooth muscle.

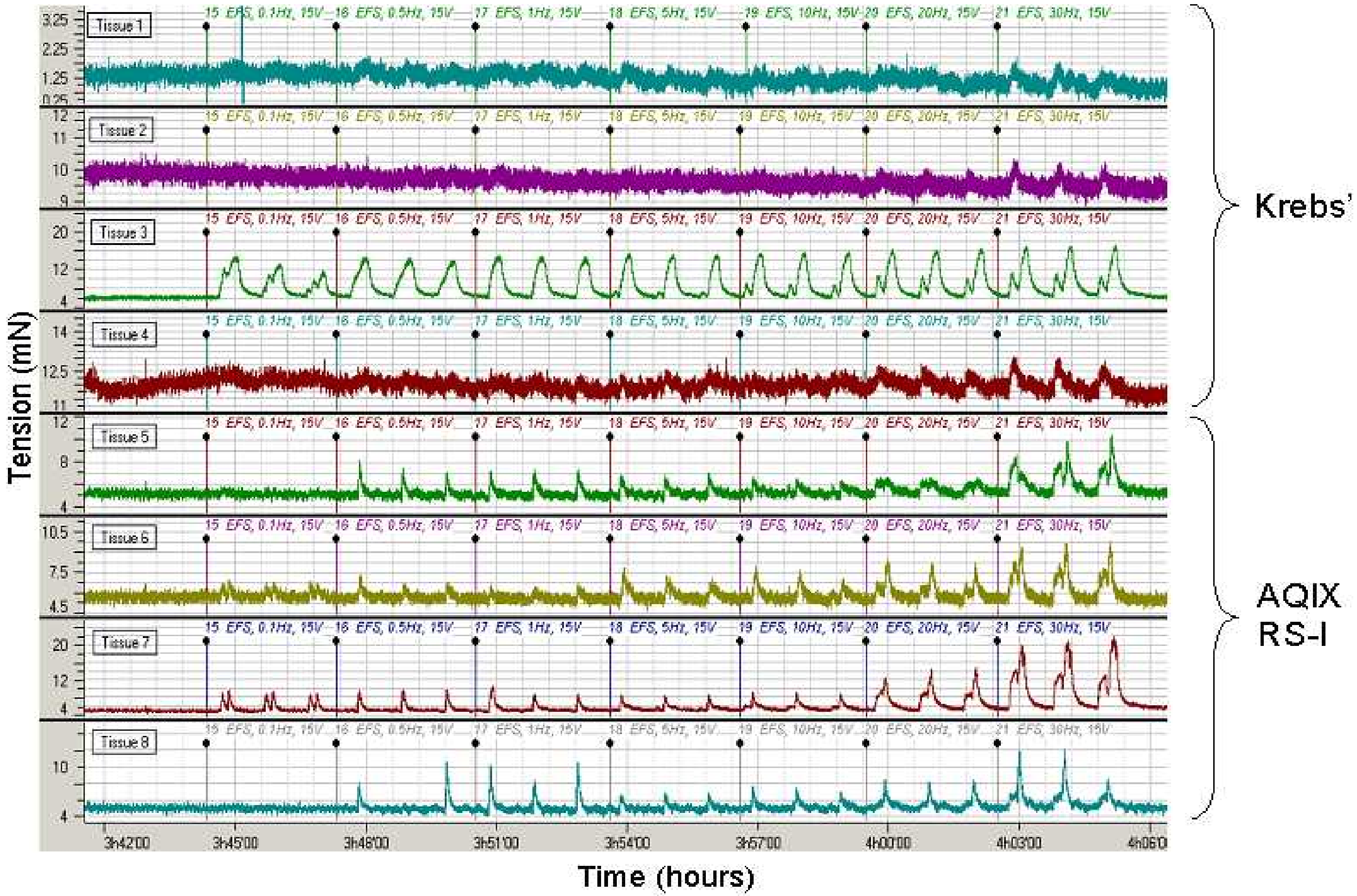


Figure 2. **Experimental traces showing EFS FRC effects on basal tone in isolated Human colon, longitudinal smooth muscle.**

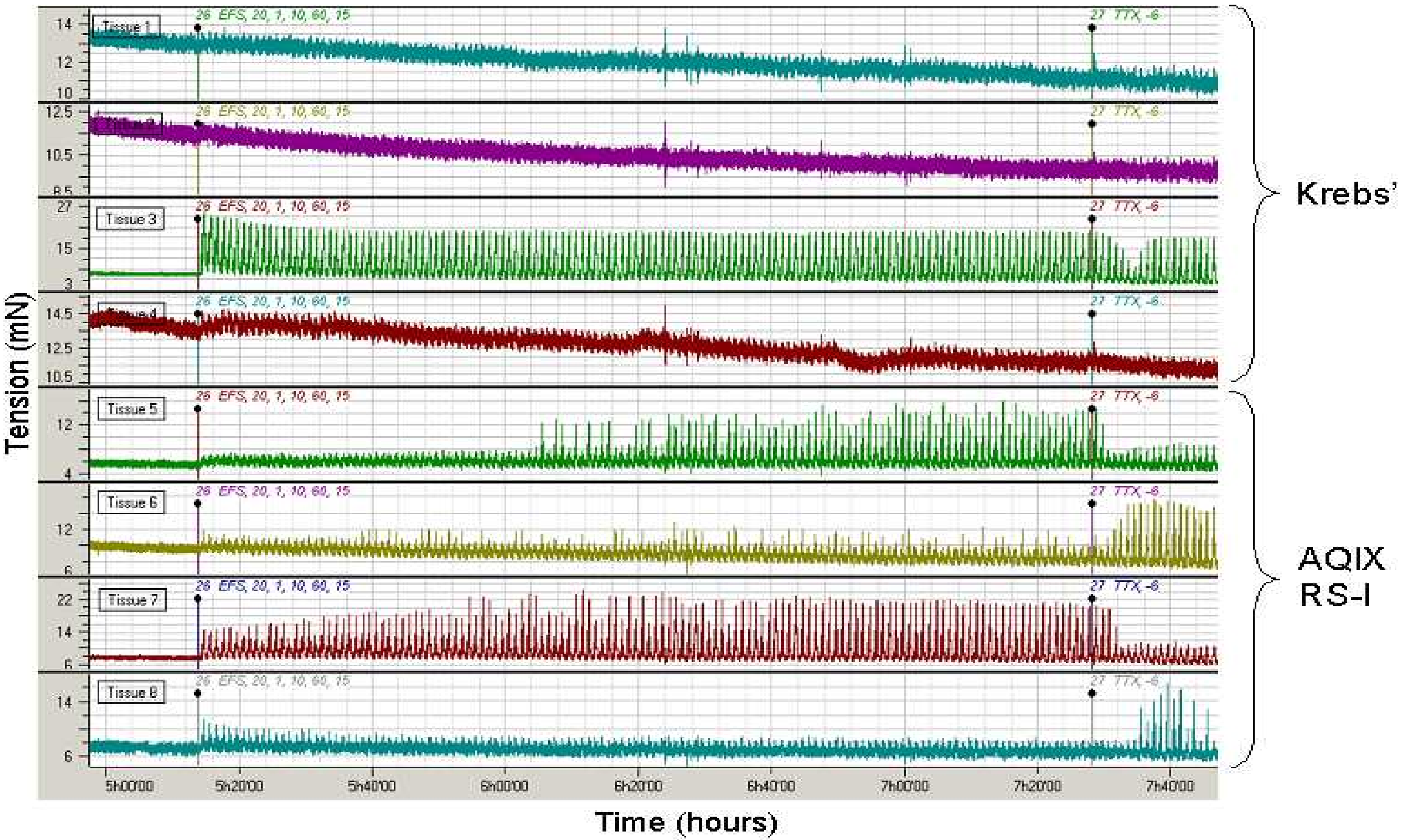
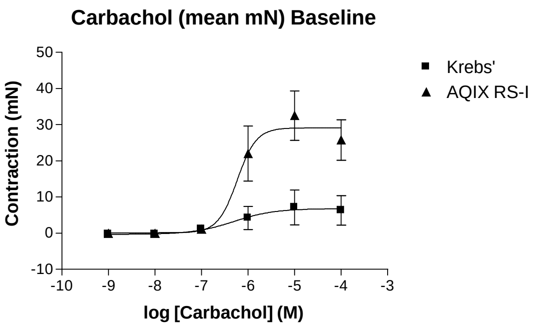
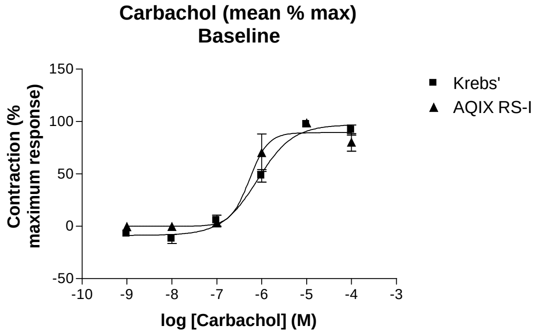
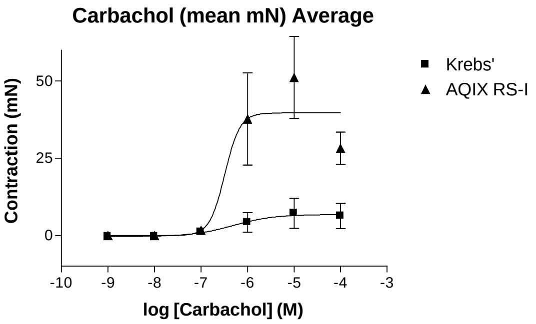


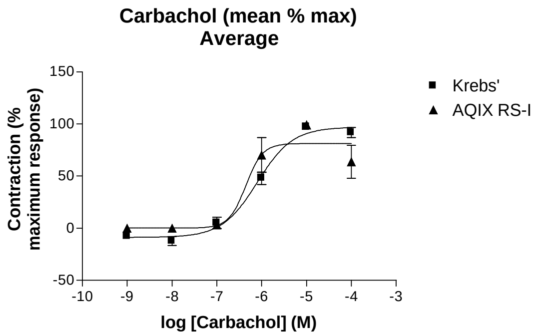
Figure 3. **Experimental traces showing EFS-evoked contractions over 2 hour period on isolated Human colon, longitudinal smooth muscle.**



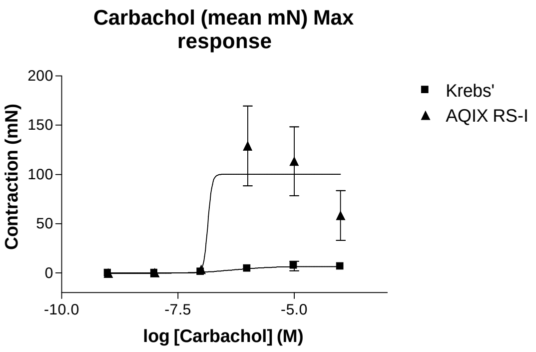


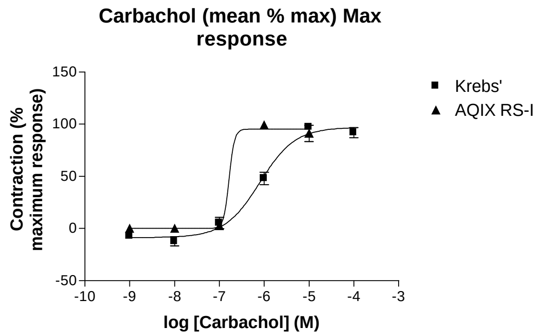
	Krebs	AQIX RS-I
LOGEC50	-6.097	-6.273
HILLSLOPE	1.101	2.098





	Krebs	AQIX RS-I
LOGEC50	-6.097	-6.355
HILLSLOPE	1.101	2.292





	Krebs	AQIX RS-I
LOGEC50	-6.097	-6.782
HILLSLOPE	1.101	7.295

Figure 4. **Effect of carbachol on isolated Human colon, longitudinal smooth muscle.** [Data are expressed as mean mN response in the left hand graphs and mean % of maximum response in the right hand graphs]

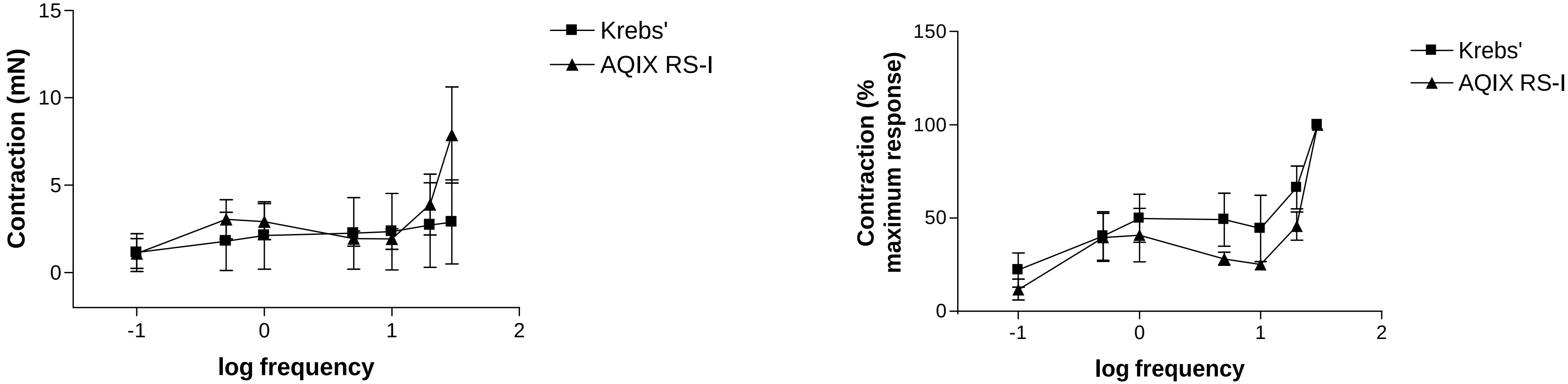


Figure 5. **Effects of electrical field stimulation (EFS) frequency-response curve on isolated Human colon, longitudinal smooth muscle.**. [Data are expressed as mean mN response in the left hand graph and mean % of maximum response in the right hand graph]

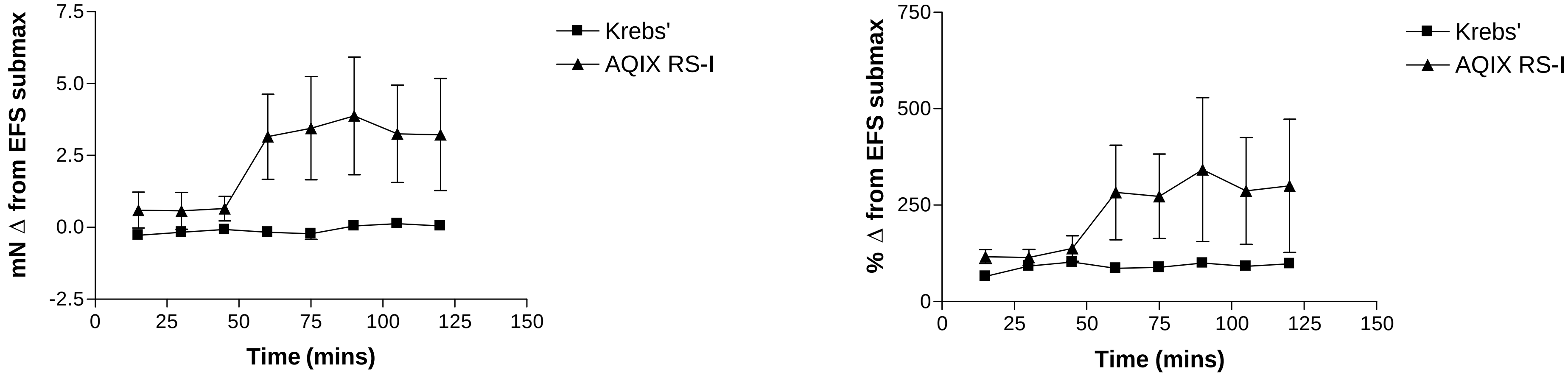
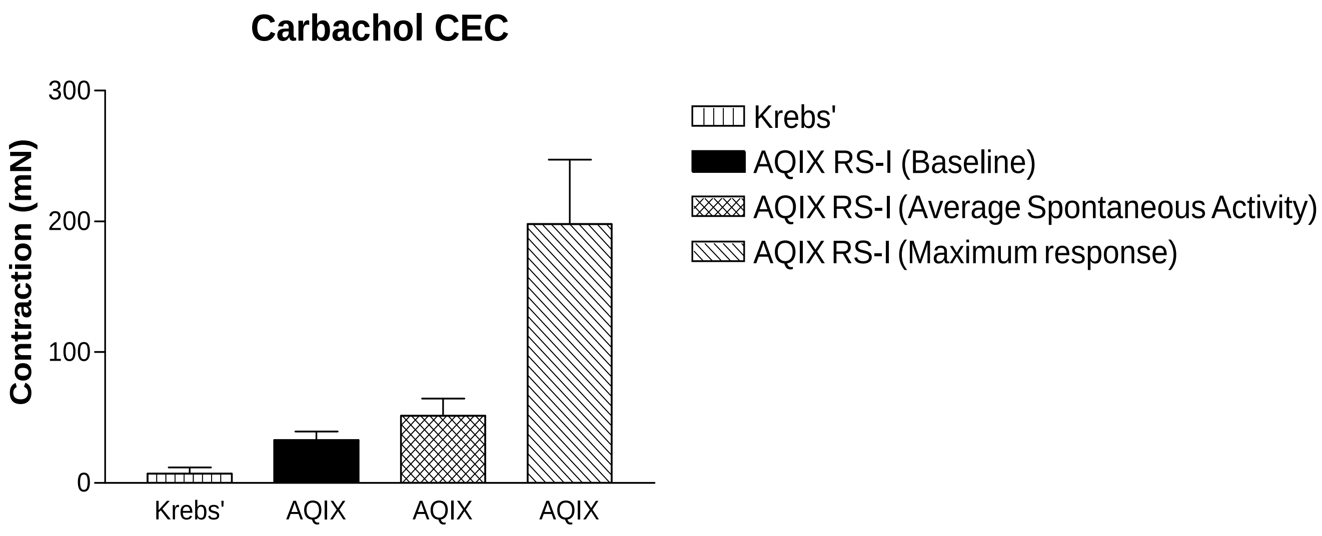


Figure 6. **Effects of EFS-evoked contractions over 2-hour time course on isolated Human colon, longitudinal smooth muscle.**

[Data are expressed as mean mN change from pre-test sub-maximal EFS response in the left hand graph and mean % change from pre-test sub-maximal EFS response in the right hand graph]



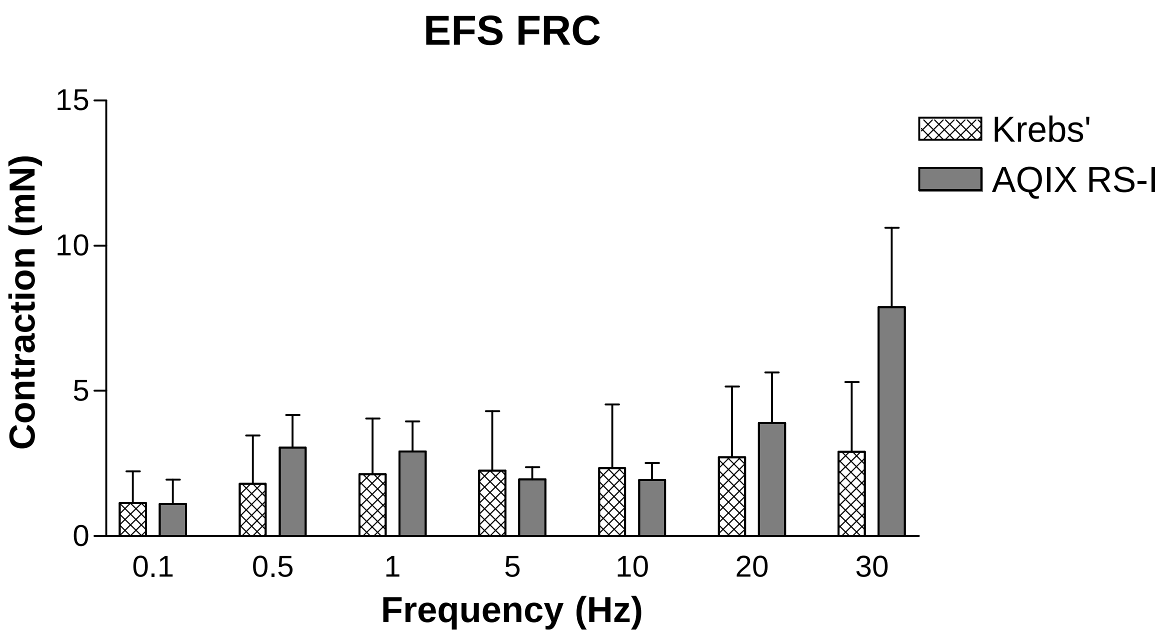


Figure 7. Carbachol concentration-effect curves and EFS FRC on basal tone in isolated Human colon, longitudinal smooth muscle.

[Data are expressed as maximum carbachol response (at the different measurement parameters) in the top histogram and mean mN response of EFS FRC in the bottom histogram]

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Inferences.

1. Clear differences were observed in the responses to carbachol between the two solutions, with much larger contractions (5x) in AQIX RS-I than in Krebs'. In addition, the carbachol responses in AQIX RS-I exhibited marked phasic contractions, whereas those in Krebs' were only tonic in nature. There appeared to be no difference in the potency of carbachol across the two groups.

2. Despite neither group eliciting clear frequency-dependant contractions during the EFS FRC, EFS-evoked contractions were more pronounced in the AQIX RS-I tissues, with all four tissues responding, whereas only one of the Krebs' tissues tissue showed a clear response. Similarly, during the 2-hour EFS period, EFS-evoked contractions were larger in magnitude in the AQIX RS-I tissues in comparison to the Krebs tissues. Additionally, there was a clear increase in contractions in two of the four AQIX RS-I tissues.

3. TTX caused contrasting effects on EFS-evoked contractions in the AQIX RS-I tissues, causing inhibition in two tissues and potentiation in the other two tissues. In the Krebs' tissues, TTX caused transient inhibition in the one clear responsive tissue.

<u>In Summary;</u> the contractions to carbachol were markedly larger, and also displayed a vastly different profile, in the AQIX RS-I perfused tissues. However, although there appeared some differences in the EFS-evoked contractions between the two tissue groups, these were less well defined.