CHAPTER IV
RESULTS OF EXPERIMENTS USING THE CYTOSENSOR® MICROPHYSIOMETER SYSTEM

4.1 Experiments using the Cytosensor® Microphysiometer System

The Cytosensor® Microphysiometer System measures the extracellular acidification rate (ECAR) of cultured cells in real time as described in Section 1.2-6. The procedure for using this system is described in Section 2.4. The system must be optimized for each cell line under study which was done for SV40-IGF-I and MAC-T cells as described in Chapter III. Maximum % ECAR peak heights were averaged and the resultant data tabulated for the experimental conditions described in this chapter.

SV40-IGF-I cells were seeded at $2x10^5$ cells/ml and MAC-T cells were seeded at $5x10^5$ cells/ml in all the experiments described in this chapter. Cells were serum-starved for 72 hr prior to the experimental runs. All % ECAR signal responses were normalized immediately prior to agent challenge, and maximum peaks for each signal were found relative to this normalization. Only the initial challenge or the initial challenge following a pre-treatment of the cells of a capsule cup membrane were included in the tabulated data. This was to avoid the complication of diminishing maximum peak heights with subsequent challenges to the same cells and to avoid data from cells that had previously been exposed to another agent than the one in question for any measurement. This eliminated the possibility of residual effects from prior challenges with different agents.

All capsule cups were seeded and incubated in Akers' Laboratory and transported to the Veterinary School the morning of the microphysiometer runs unless otherwise indicated. In the later studies, cell cultures were maintained at the Veterinary School to avoid any undue stress to the cells as a result of transportation.

4.1.1 Responses in % ECAR of SV40-IGF-I and MAC-T Cells to IGF-I

SV40-IGF-I and MAC-T cells of different passage numbers and during different experimental runs were challenged for 14 min with different concentrations of IGF-I as shown in Table 4.1. Standard errors are reported for averages of two or more peak values.

The MAC-T cells responded with lower % ECAR when tested in parallel runs with SV40-IGF-I with IGF-I challenge concentrations of 2 and 5 ng/ml in the initial experimental runs. Both cell lines were maintained at a different site from the microphysiometer test site during these runs. It was noted at this time that MAC-T cells responded very poorly after having been deprived of CO_2 and when many enlarged cells could be seen in the tissue culture plate. In both instances, a new culture of MAC-T cell was started from a vial in nitrogen storage. The SV40-IGF-I cells did not seem to be affected in this same way. Cells were later maintained at the test site when it was suspected that environmental stress may have had an effect on the MAC-T cells during transport between buildings. The data for MAC-T cells challenged with 50 ng/ml had the same average % ECAR (125.7) as SV40-IGF-I cells challenged on two channels in a parallel test run after being maintained at the test site. This suggested that the SV40-IGF-I cells were less vulnerable to environmental stress compared to the MAC-T cells. The reported average value for SV40-IGF-I cells challenged with 50 ng/ml IGF-I in Table 4.1 is 122.4 ± 4.8 which is the averaged data for 5 data points including 2 points from cells maintained at the same site as the microphysiometer.

To determine if there was a significant difference in the IGF-I response data for the SV40-IGF-I and MAC-T cells maintained in a different building prior to testing, the data for % ECAR for 2 and 5 ng/ml IGF-I stimulation were compared using the ANOVA test (that the two groups are identically distributed) with StatXact software. Data for % ECAR for 50 ng/ml IGF-I was omitted because the cells were maintained at the same site and had the same average % ECAR. Using the two-sided test, the p-value is 0.073 which

Table 4.1 Response of Cells to IGF-I

IGF-I Concentration (ng/ml)	Maximum % ECAR ± Standard Error	
	SV40-IGF-I	**MAC-T**
0.1	102.0 ± 0.2***	
0.5	112.6 ± 2.0	
1	108.7 ± 0.1***	
2	116.6 ± 1.3	103.0 ± 0.5***
3	117.6**	
5	119.1 ± 1.7	111.7 ± 0.3****
10	115.7 ± 1.7***	
50	122.4 ± 4.8	125.7 ± 0.2*
100	115.3**	
500	109.0**	

* Average of 2 data points taken with MAC-T cells maintained at test site where they did not undergo environmental stress as did the other MAC-T cells tested.

** Data from a single data point.

*** Data from the average of two data points

**** Data from the average of three data points.

All other values are averages of five to nine data points with standard errors reported.

is borderline in significance. The small sample size was likely the reason that the p-value was not ≤ 0.05.

To determine a dose response of SV40-IGF-I cells to IGF-I, a logarithmic graph of the averaged data is shown in Figure 4.1. Data are plotted for the points from 0.1 to 50 ng/ml IGF-I. Data for 100 ng/ml and 500 ng/ml were not included since they show a decline in

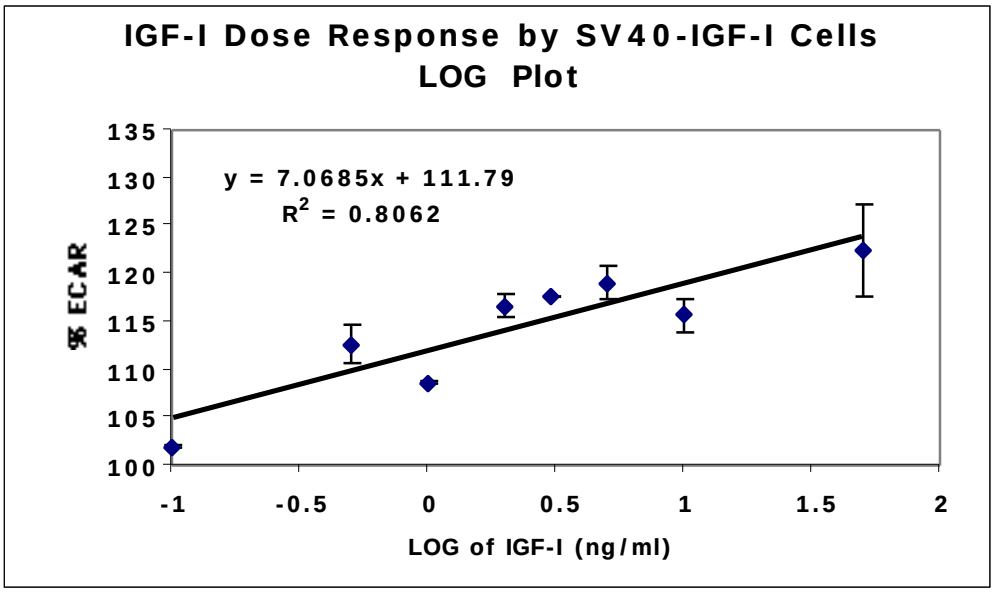

Figure 4.1 *Logarithmic plot of dose dependence of IGF-I on % ECAR. Points at IGF-I concentrations 0.5, 2, 5, and 50 ng/ml IGF-I are the averages of at least 5 data points. Error bars are for the standard error. Significance F for the regression is 0.0025. The slope 7.0685 has a p-value of 0.0025 and the intercept 111.79 has a p-value of $1.05x10^{-10}$. All are clearly below the significance requirement of 0.05 or less.*

response, possibly due to saturation or diminished reponse at these high values. However only one data point was taken at each of these high concentrations and more replications must be made to verify the behavior.

Regression analysis of the plot % ECAR *vs*. the LOG of the IGF-I concentration (Figure 4.1) resulted in the regression equation y = 7.0685x +111.79 where y = % ECAR and x = LOG (IGF-I concentration) with R^2 = 0.8062. Overall it can be seen that the response is increasing in a logarithmic manner from 0.1 to 50 ng/ml IGF-with regression analysis using Microsoft® Excel. The regression significance F of the plot using the two sided test for ANOVA is 0.0025. The p-value for the slope 7.0685 is 0.0025 and the p-value for the intercept 111.79 is 1.05×10^{-10}. These significance values are all under 0.05. This verifies that the slope of the trendline is positive for cell stimulation by IGF-I in the range of 0.1 to 50 ng/ml IGF-I.

4.1.2 Responses in % ECAR of SV40-IGF-I cells to R^3-IGF-I

The response of SV40-IGF-I cells to 5 ng/ml IGF-I was compared to 5 ng/ml R^3-IGF-I in Table 4.2. Reported maximum % ECAR values are the average of 3 channels. R^3-IGF-I

Table 4.2 Response of SV40-IGF-I cells to IGF-I or R^3-IGF-I

Growth Factor	Maximum % ECAR ± Standard Error
5 ng/ml IGF-I	117.9 ± 0.1
5 ng/ml R^3-IGF-I	119.7 ± 0.5
Control	101.8 ± 0.6

Reported maximum % ECAR for the growth factors are the averages from three channels. The control was not stimulated by any growth factor and is the average from two channels. The value above 100 for the control reflects a typical upward trend of the ECAR for living cells on the microphysiometer.

is an analogue of IGF-I and binds IGFBP-3 with a lower affinity than IGF-I. A two-sided ANOVA test of the data was performed using StatXact software. The resulting p-value is 0.05, so that the % ECAR response of IGF-I and the analogue R^3-IGF-I shows borderline significant difference between the two growth factors. No significant difference implies that endogenous IGFBP-3 from the SV40-IGF-I cells does not affect the % ECAR response. This implies that the binding of IGF-I to the cell surface receptor is not affected under these experimental concentrations providing signaling is directly related to binding. To compare the two growth factors, each was purchased from the same company, packaged in identical containers and reconstituted identically so that the possibility of error from slight variation in reconstituted concentration would not be a factor. Figure 4.2 shows how peak shapes were identical for the two growth factors.

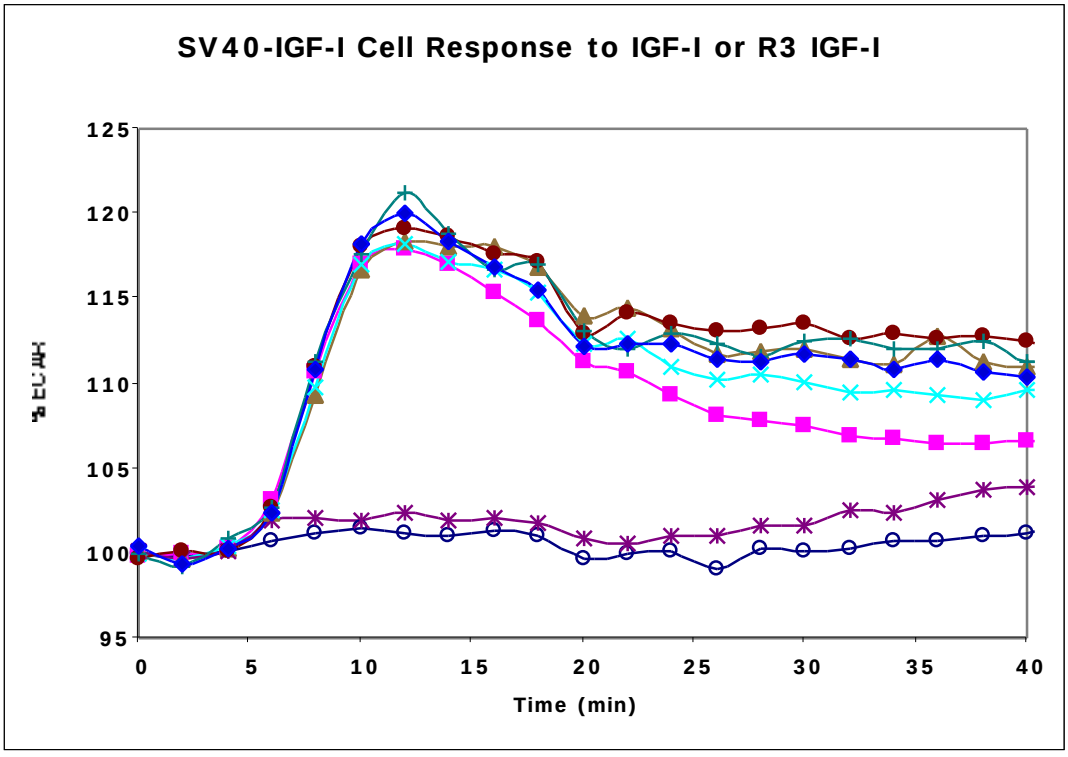

Figure 4.2 *SV40-IGF-I cell reponses to 5 ng/ml IGF-I (■ ▲ ✖) or R^3IGF-I (● ❙ ◆). Control channels received no growth factor (✳ ❍). % ECAR response is normalized prior to stimulation. Peak values are tabulated in Table 4.3.*

4.1.3 Response in % ECAR of SV40-IGF-I cells to IGFBP-3 alone

SV40-IGF-I exposed to IGFBP-3 alone challenges of 0.5, 5, and 50 ng/ml did not show an increase or decrease in % ECAR as seen in Table 4.3 and the first 22 min of the graph in Figure 4.3.

Table 4.3 Response of SV40-IGF-I cells to IGF-I and IGFBP-3

Growth Factor	IGFBP-3 Preincubation	IGFBP-3 Concentration	Maximum % ECAR ± Standard Deviation
none	yes	0.5 ng/ml	100.3 *
none	yes	5 ng/ml	102.6 ± 0.9
none	yes	50 ng/ml	102.6 ± 0.4
5 ng/ml IGF-I	0.5 ng/ml	none	116.9 *
5 ng/ml IGF-I	5 ng/ml	none	118.0 ± 3.3
5 ng/ml IGF-I	50 ng/ml	none	116.6 ± 1.6

* Data represents a single point. All other points are averages of two points.

Regression analysis of the response by SV40-IGF-I cells to IGFBP-3 alone shows no upward trend with increased dose of IGFBP-3 from 0 to 50 ng/ml IGFBP-3. Regression analysis was performed using Microsoft® Excel for the resultant equation % ECAR = 0.0236 x (IGFBP-3, ng/ml) + 101.55 with $R^2 = 0.27$. Data points are the average of two measurements except for 0.5 ng/ml which is from a single measurement. Significance F

for the regression is 0.48, and the p-value for the slope is 0.48 and for the intercept is $4.5x10^{-5}$. This rejects the computed slope as significant compared to zero.

4.1.4 Responses in % ECAR of SV40-IGF-I Cells to IGF-I Alone after Preincubation with IGFBP-3 Alone

Figure 4.3 shows no effect on the peak stimulation response of SV40-IGF-I cells by IGF-I alone for 14 min IGF-I after preincubation of the cells with different concentrations of

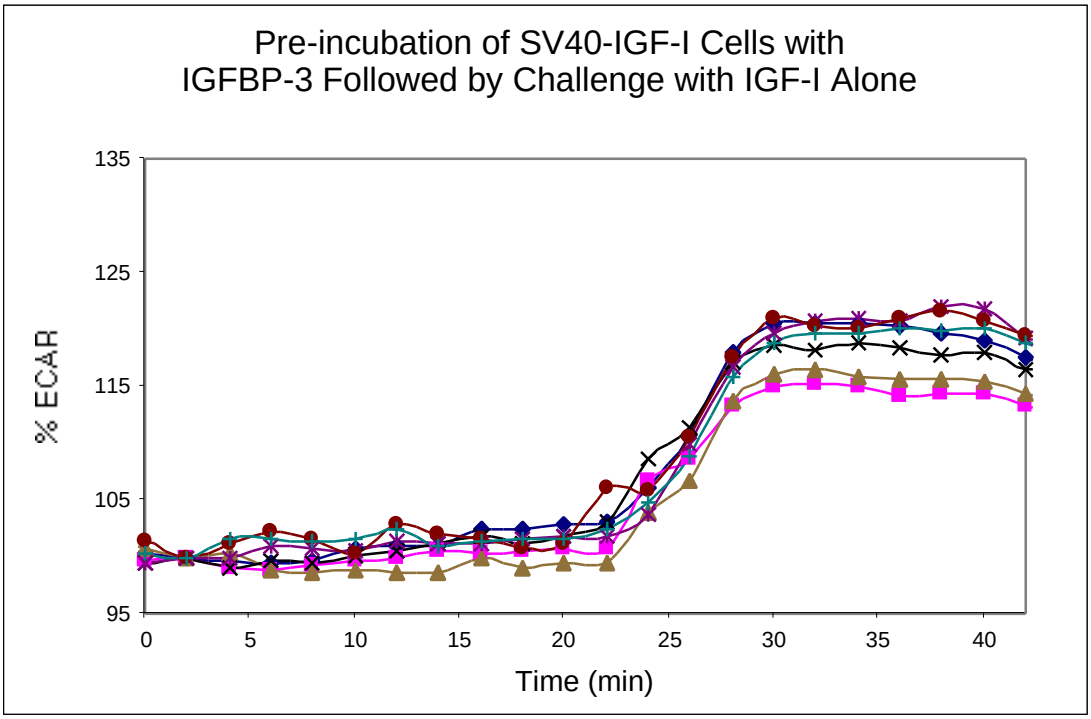

Figure 4.3 *SV40-IGF-I cells were stimulated at 22 min by 5 ng/ml IGF-I after pre-incubation with varying doses (Control (◆, ■), 0.5 ng/ml (▲), 5 ng/ml (×, ✳), and 50 ng/ml (● l)) of IGFBP-3. Data was normalized prior to IGFBP-3 challenge. Data was not re-normalized at 22 min when IGFBP-3 was discontinued and IGF-I challenge began.*

IGFBP-3. Each data point in Table 4.3 is the average of two points except for 0.5 ng/ml IGFBP-3 which is one point. Linear regression of the data was performed using Microsoft® Excel for the resultant equation % ECAR = -.005 x (IGFBP-3, ng/ml) + 116.91 with $R^2 = 0.021$. Analysis of the resultant regression curve shows significance F to be 0.86, rejecting the slope as significant compared to zero. Consequently, IGF-I stimulation of SV40-IGF-I cells shows no effect from the preincubation of cells with IGFBP-3. Because there was no residual effect from the pre-incubatin on the IGF-I stimulation, it is possible that there was no binding or downregulation by IGFBP-3 in this time frame.

4.2 Preliminary Experiments Using the Cytosensorâ Microphysiometer System

The following experiments are only preliminary, but suggestive, as sufficient repetitions or adjustment of challenge concentrations and/or agents have not yet been conducted. Cells were seeded at densities determined previously ($2x10^5$ cells/ml for SV40-IGF-I cells and $5x10^5$ cells/ml for MAC-T cells). Cells were serum starved for 72 hrs prior to a run, and % ECAR was normalized prior to the first agent challenge.

4.2.1 SV40-IGF-I Dose Response to Exogenous IGFBP-3 Coincubated with 3 ng/ml IGF-I

To determine a dose response of SV40-IGF-I cells to exogenous IGFBP-3, cells were challenged for 14 min by 3 ng/ml IGF-I with 200, 100, 50, 10, 5, 0.5, or 0 ng/ml IGFBP-3. Dilutions of IGF-I and IGFBP-3 were prepared separately during the cell equilibration period of one hour and then mixed just prior to loading onto the microphysiometer. Peak % ECAR responses are listed in Table 4.4. The listed values for maximum % ECAR are the averages of duplicate channels with two exceptions: for the IGFBP-3 concentration of 0 ng/ml the maximum % ECAR is the average of 3 channels and for the IGFBP-3 concentration of 5 ng/ml the maximum % ECAR is that of a single channel. This is only preliminary data. As can be seen from the Table 4.4, there is an apparent peak between 5

and 10 ng/ml which may not be significant. More repetitions are necessary to determine the dose response.

TABLE 4.4 Dose Dependent Response of SV40-IGF-I Cells to Exogenous IGFBP-3 with Coincubation of 3 ng/ml IGF-I

IGFBP-3 Concentration	Maximum % ECAR ± Standard Deviation
200 ng/ml	109.7 ± 1.1
100 ng/ml	109.2 ± 2.4
50 ng/ml	110.9 ± 3.3
10 ng/ml	117.2 ± 3.6
5 ng/ml	113.0*
0.5 ng/ml	111.9 ± 2.1
0 ng/ml	115.7 ± 1.7

**Data from one data point. All other values (except for 0 ng/ml which is the average of three data points) are the average of two measurements.*

The data shows a dose response of diminishing peak with increasing concentration of IGFBP-3 from 10 to 200 ng/ml IGFBP-3. Additional repetitions are necessary to verify the behavior at lower concentrations of IGFBP-3 for stimulation by 3 ng/ml IGF-I.

4.2.2 Effect of SV40-IGF-I Proteoglycan on IGF-I Response by SV40-IGF-I Cells

SV40-IGF-I cells (passage 6) were challenged in duplicate with 3 ng/ml IGF-I in the presence of 1 ug/ml SV40-IGF-I proteoglycan prepared from conditioned media as described in Sections 2.5 through 2.9. For controls, cells were challenged in 2 channels

with 3 ng/ml IGF-I alone, in two channels without proteoglycan or IGF-I, and in one channel with 1 ug/ml proteoglycan alone.

As can be seen from the resulting graph in Figure 4.4, additional studies at different concentrations of proteoglycan must be made to reach conclusive results. It appears that at 1 ug/ml, the proteoglycan may increase IGF-I binding. Comparison of one channel of proteoglycan with the two channels carrying neither IGF-I nor proteoglycan shows a depressed response signal for proteoglycan alone. This, however, is a single point, so additional runs are clearly needed to draw any conclusions from this experiment.

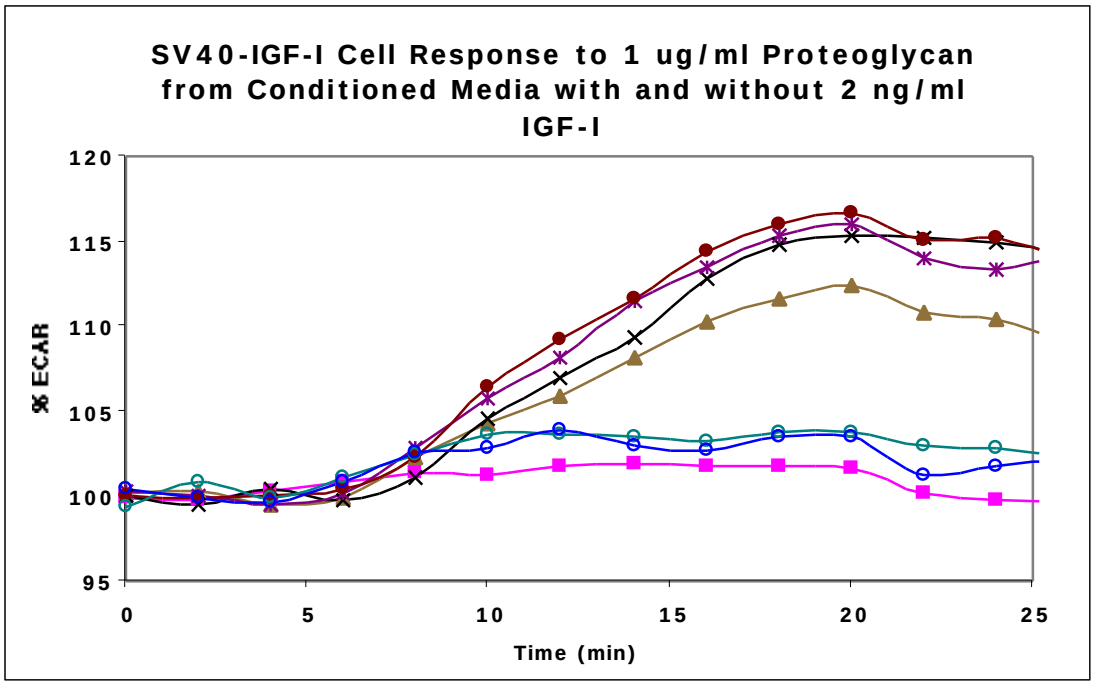

Figure 4.4 *% ECAR response by SV40-IGF-I cells (passage 6) to 2 ng/ml IGF-I in the presence of 1 ug/ml SV40-IGF-I proteoglycan (● ✳). Control channels are challenged with PG alone (■), IGF-I alone (▲ ✖), or neither agent (❍ ❍). % ECAR is normalized prior to challenges.*

4.2.3 Amiloride Inhibition of the Na+/H+ Exchanger

Different agents which are specific in the inhibition of proton release may be used during the challenge of cells on the microphysiometer to confirm the source of the extracellular acidification. Amiloride is a known inhibitor of the Na^+/H^+ exchanger and when administered to the cells 30 min prior to and at the same time as a stimulating factor, it will reduce the % ECAR response by the cells (Molecular Devices, 1996). To support that the IGF-I was attaching to the IGF-I receptor and initiating a signal cascade that included the release of protons through the Na+/H+ exchanger, the cells were equilibrated in 100 µM amiloride for 30 min. Control cells did not receive amiloride. Cells were then challenged for 14 min with 50 ng/ml IGF-I. Channels with amiloride continued to receive the amiloride with the IGF-I challenge.

Table 4.5 shows the results for maximum % ECAR for SV40-IGF-I (passage 6) and MAC-T cells (passage 6) with and without 100 M amiloride during stimulation with 50 ng/ml IGF-I. Each condition was run in duplicate, and the reported value for each

Table 4.5 Decreased Response to 50 ng/ml IGF-I by SV40-IGF-I and MAC-T Cells when Treated with 100 uMof Amiloride

Cell Line	Maximum % ECAR ± Standard Deviation	
	100 uM Amiloride	No Amiloride
SV40-IGF-I	118.3 ± 4.0	125.7 ± 2.3
MAC-T	121.1 ± 1.3	125.7 ± 0.2

condition is the average. The graphical results in figures 4.5 and 4.6 show a depression in response to IGF-I for both cell lines in the presence of amiloride. Analysis using StatXact software resulted in a p-value of 0.33 using a one sided ANOVA since

inhibition with amiloride was expected. The p-value is too high to consider the difference as significant. This is likely due to the very small sample size. Additional experiments will need to be run to verify the effect of amiloride. Either a larger sample size for the data analysis or an adjustment of concentrations (less IGF-I or more amiloride) is needed to reveal a decreased % ECAR as a result of amiloride addition.

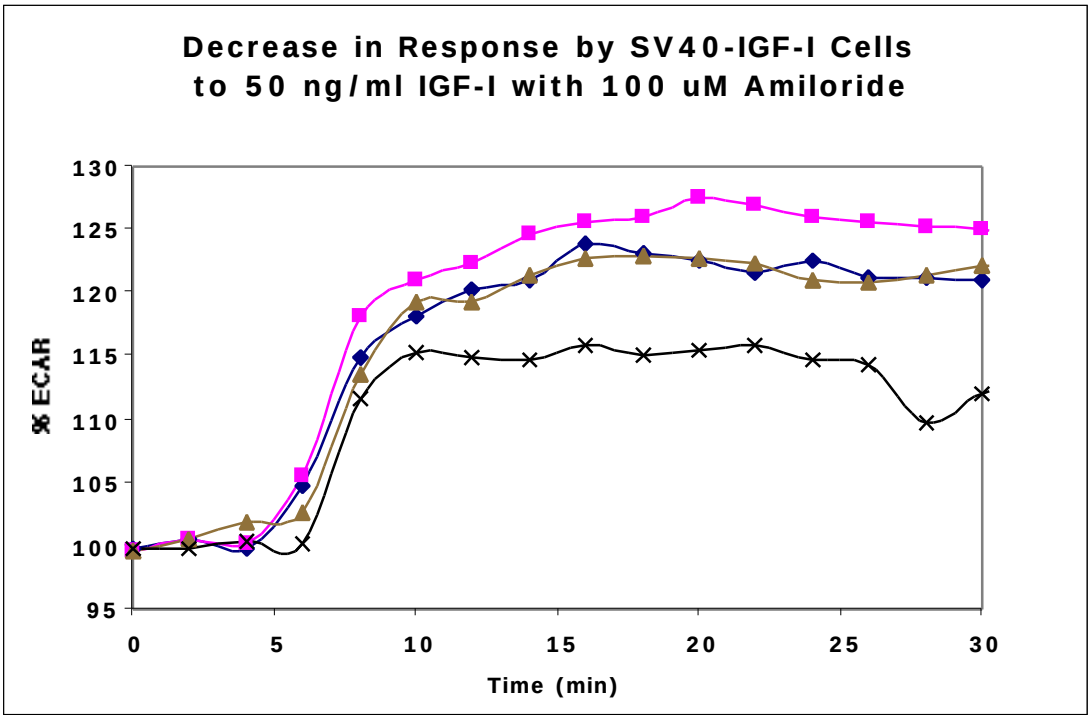

Figure 4.5 *SV40-IGF-I cells (passage 6) stimulated for 14 min by 50 ng/ml IGF-I alone (■ ◆) and in the presence of amiloride (▲✖). IGF-I challenges began at 4 min on the graph. Cells receiving amiloride were preincubated in 100 µM amiloride for 30 min prior to the IGF-I challenge and continued to receive amiloride during the challenge. Control cells did not receive amiloride.*

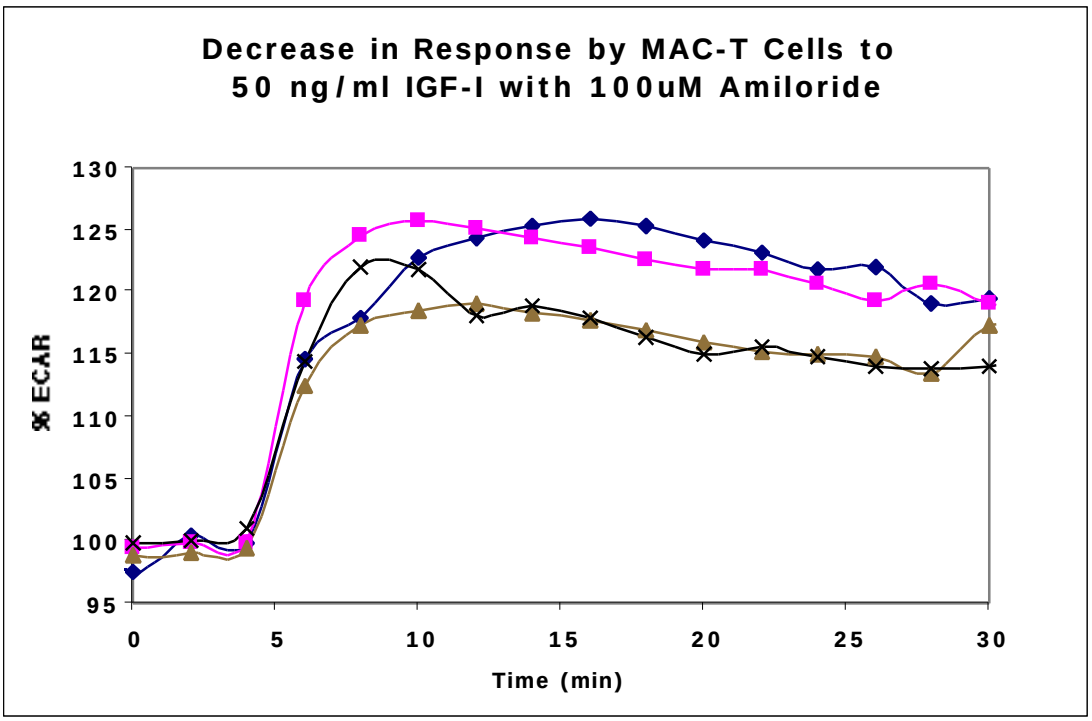

Figure 4.6 *MAC-T cells (passage 6) stimulated for 14 min by 50 ng/ml IGF-I alone (■◆) and in the presence of amiloride (▲✖). Challenges began at 4 min on the graph. Cells receiving amiloride were preincubated in 100 µM amiloride for 30 min prior to the IGF-I challenge and continued to receive amiloride during the challenge. Control cells did not receive amiloride.*