Increased evidence indicates that ion channels and transporters cooperate in regulating different aspects of tumor pathophysiology. In cancer cells, H+/HCO_3^- transporters usually invert the transmembrane pH gradient typically observed in non-neoplastic cells, which is thought to contribute to cancer malignancy. To what extent the pH-regulating transporters are functionally linked to K^+ channels, which are central regulators of cell membrane potential (V_m), is unclear. We thus investigated in colorectal cancer cells the implication of the pH-regulating transporters and KV11.1 (also known as hERG1) in the pH modifications stimulated by integrin-dependent cell adhesion. Colorectal cancer cell lines (HCT 116 and HT 29) were seeded onto β1 integrin-dependent substrates, collagen I and fibronectin. This led to a transient cytoplasmic alkalinization, which peaked at 90 min of incubation, lasted approximately 180 min, and was inhibited by antibodies blocking the β1 integrin. The effect was sensitive to amiloride (10 µM) and cariporide (5 µM), suggesting that it was mainly caused by the activity of the Na+/H+ antiporter NHE1. Blocking KV11.1 with E4031 shows that channel activity contributed to modulate the β1 integrin-dependent pHi increase. Interestingly, both NHE1 and KV11.1 modulated the colorectal cancer cell motility triggered by β1 integrin-dependent adhesion. Finally, the β1 integrin subunit, KV11.1 and NHE1 co-immunoprecipitated in colorectal cancer cells seeded onto Collagen I, suggesting the formation of a macromolecular complex following integrin-mediated adhesion. We conclude that the interaction between KV11.1, NHE1, and β1 integrin contributes to regulate colorectal cancer intracellular pH in relation to the tumor microenvironment, suggesting novel pharmacological targets to counteract pro-invasive and, hence, pro-metastatic behavior in colorectal cancer.

Keywords: hERG1, integrins, Collagen I, beta 1 integrin subunit, cariporide, lateral motility
INTRODUCTION

Ion channels and transporters are progressively emerging as pivotal modulators of different aspects of cancer cell behavior (Arcangeli et al., 2009; Lastraïoli et al., 2015a). Such pleiotropic effects can be traced back to the regulation of either $V_m$ (an effect mainly exerted by K+ channels; Huang and Jan, 2014), or of the concentration and intracellular distribution of specific ion species, such as Ca$^{2+}$ (Bose et al., 2015) and H+ (Gadsby, 2009), or to the direct modulation of intracellular signaling pathways (Arcangeli et al., 2009; Becchetti et al., 2019). However, the molecular interactions between these mechanisms are poorly understood, and a unified picture of the cancer cell pathophysiology is still missing.

One of the K+ channels most often dysregulated in cancer is Kv 11.1 (or hERG1), which regulates the resting $V_m$ in excitable cells (Bauer and Schwarz, 2018), as well as in cancers arising from excitable (e.g., neuroblastomas, Crociani et al., 2003) and non-excitable tissues. In particular, Kv 11.1 modulates intracellular signaling pathways triggered by integrin-mediated adhesion, both in leukemias (Pilolzi and Arcangeli, 2009) and solid cancers such as colorectal (Crociani et al., 2013), pancreatic (Lastraïoli et al., 2015b), gastric (Crociani et al., 2014) and mammary (Becchetti et al., 2017). The underlying mechanism involves the formation of a macromolecular complex between Kv 11.1 and β1-integrins, which promotes angiogenesis and triggers metastatic spread (Crociani et al., 2013; Becchetti et al., 2017). In pancreatic ductal adenocarcinoma cells, this occurs through the regulation of f-actin dynamics in filopodia (Manoli et al., 2019).

Cancer proliferation and migration are also controlled by intracellular pH, whose regulation is frequently dysregulated in tumors (Webb et al., 2011). Hence, targeting the pH regulating transporters has been suggested as a therapeutic strategy (Persi et al., 2018). Cancer cells generally display a higher activity of the V-type H+-ATPases expressed on the plasma membrane (Sennoune et al., 2004), the Na+/H+ exchanger NHE1 (Stock and Pedersen, 2003; Stock et al., 2005; Stock et al., 2008). The corresponding local pH increase stimulates the focal adhesion dynamics. First, it supports the F-actin severing activity of coflin (Frantz et al., 2008), which produces free-barbed-end actin in the lamellipodium. Second, it reduces the affinity of talin for actin (Srivastava et al., 2008). It is thus clear that the pH regulating mechanisms are essential determinants of the tumor microenvironment and the cancer cell crosstalk.

Based on the above premises, we investigated whether the pH-regulating transporters are functionally linked to Kv 11.1 channels, which are strongly dysregulated in cancer cells, and whose activity is tightly related to integrin receptors in modulating cancer cell proliferation and migration (Becchetti et al., 2019). In particular, we studied if pH regulating mechanisms provide a direct link between integrin-mediated hERG1-dependent cell adhesion and the tumor microenvironment. As a model, we used ColoRectal Cancer (CRC) cells, in which knowledge about Kv 11.1 physiology is particularly extensive.

MATERIALS AND METHODS

Unless otherwise indicated, chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, USA).

Cell Lines and Cell Culture

The human colon carcinoma cell lines HCT 116 and HT 29 were cultured at 37°C and 5% CO₂ in air, in Roswell Park Memorial Institute (RPMI) 1640 Medium, with sodium bicarbonate (2 g/L) and 2 mmol/L L-glutamine (“culture medium”), supplemented with 10% fetal bovine serum (FBS) (Euroclone, Italy). In all experiments, cells were starved overnight in culture medium without serum (“no-serum medium”) and detached, prior to experiment, with PBS plus 5 mM EDTA.

Coating of Culture Substrates

Extracellular Matrix (ECM) proteins at the final amount per cm² of surface area shown in brackets: Fibronectin (FN, 5 μg), Collagen-1 [Col-1, 10 μg; produced as reported in Hallowes
et al. (1980)], Vitronectin [VN, 0.5 µg; produced as reported in Yatohgo et al. (1988)]. Coating with Polyllysine (PL, 0.1 µg) was taken as a control of integrin-independent adhesion, while seeding onto uncoated dishes was our “no-adhesion” control. FN and VN were diluted in PBS, Col-1 in serum-free media, PL in bi-distilled water and plated to cover the entire growth surface, followed by 1 h incubation at either 37°C (for Col-1) or room temperature (for FN, VN and PL). After the coating procedure, incubation with Bovine Serum Albumin (BSA) for 15 min at 37°C was performed to block all the uncovered plastic sites.

**Measurement of pHi**

To determine pHi, we used 2′,7′-Bis (2-carboxyethyl)-5 (6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM). Cells were starved and detached as described above, and seeded (5 x 10⁴ cells/well) in no-serum medium onto uncoated or coated (see above) 96-well plates (clear bottom 96-well plate, polystyrene, TC-treated, clear flat bottom wells, sterile, w/lid, black; Corning, New York, USA). Cells were then incubated at 37°C in 5% CO₂ for different times, in the absence or presence of different drugs (see below). At selected time points, the medium was removed and BCECF-AM (1µM final concentration in loading solution (HBSS 1X plus 0.01% NaHCO₃, pH 7)) was added for 30 min at 37°C and 5% CO₂. After incubation, cells were washed twice with loading solution at room temperature. For measurement of initial (time 0) pHi, cells were detached, kept in suspension in a 1.5 ml tube and incubated in BCECF-AM-containing solution for 30 min at 37°C. Next, they were washed twice with loading solution, poured in a 96-well plate at 5x10⁴ cells/well at room temperature, and immediately transferred to the microplate reader. Fluorescence intensity was immediately measured with a microplate reader (Infinite 200 PRO, Tecan, Switzerland) set at the following wavelengths: 440–490 nm for excitation and 535 nm for emission. A calibration curve was set up using a high K⁺/Nigericin solution (135 mM KCl, 2 mM K₂HPO₄, 20 mM HEPES, 1.2 mM CaCl₂ and 0.8 mM MgSO₄) in a range of pH from 5.0 to 8.5. All pH values were calculated using 490/440nm fluorescence ratio and applying standard curve and linear equations, as detailed in Grant and Acosta, 1997.

**Modulators of β1 Integrin-Mediated Adhesion**

The mouse monoclonal anti-β1 integrin antibody BV7 (anti-β1 Ab, kindly gifted by Prof. P. Defilippi, University of Turin, Italy) (Martin-Padura et al., 1994) was used to block β1-integrins as described in Hofmann et al. (2001). Briefly, cells were seeded onto Col-I in no serum medium, containing anti-β1 Ab (20 µg/ml), for 90 min at 37°C and 5% CO₂. Cells (mostly detached) were collected, and pH measurement was performed as above described for time zero condition.

**Modulators of pH-Regulating Transporters and Kv11.1**

We used the following compounds: 100 µM acetazolamide (CA inhibitor; Parkkila et al., 2000), 10 µM amiloride (inhibitor of NHE1 and epithelial Na⁺ channel, ENaC; Masereel et al., 2003), 5 µM cariporide (specific NHE1 inhibitor; Hulikova et al., 2013), 30 µM S0859 (NBC inhibitor; Hulikova et al., 2013), and 40 µM E4031 (Tocris, Bristol, UK; Kv11.1 blocker; Masi et al., 2005).

Drugs were added to the cells seeded on uncoated or coated surfaces at different time points. Preliminarily, all compounds were tested for their potential cytotoxic effects at all the used concentrations, by measuring cell viability with the trypan blue test (Pillozzi et al., 2018), at 30, 90 and 180 min of incubation. None of the modulators had any cytotoxic effect (Table 1).

**Lateral Motility Assay**

Lateral motility was determined using 35 mm dishes and drawing 15 horizontal lines and 3 perpendicular lines on the dish bottom to generate a grid system. Plates were coated with Col-I and 5x10⁵ cells were seeded and allowed to attach for 90 min. Three wounds were drawn following the 3 horizontal lines. Subsequently, the following treatments were performed with drugs diluted in RPMI medium: control, E4031 40 µM, E4031 40 µM + cariporide 5 µM. Then, the distances between cells were measured at each mark point (where the 3 horizontal lines crossed the 15 vertical lines) using a light microscope. The widths measured at time 0 correspond to the W₀ parameter. These different 45 points were measured again after 90’. Motility Index (MI) was assessed using the following formula: MI = 1 – W₀/W₉₀, where W₀ is the width of the wounds after 90’.

Each treatment was performed in triplicate and the experiments were carried out at least 3 times.

**Co-Immunoprecipitation Experiments**

For (co)-immunoprecipitation experiments cells were seeded and incubated on Col-I. Cells were gently collected by mild scraping and resuspended in ice cold PBS. Protein extraction, quantification and total lysisate incubation with protein A/G agarose beads (Santa Cruz Biotechnology, Texas, USA) were performed as previously reported (Becchetti et al., 2017). In particular, the composition of the lysis buffer was the following:

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**Table 1** | Percentage of alive cells after ICT modulators treatment for 30, 90, and 180 min (± s.e.m.).

|          | HCT 116 | HT 29 | HCT 116 | HT 29 | HCT 116 | HT 29 |
|----------|---------|-------|---------|-------|---------|-------|
| Acetazolamide | 99.01 ± 0.004 | 99.02 ± 0.004 | 98.02 ± 0.003 | 99.01 ± 0.009 | 97.02 ± 0.004 | 96.03 ± 0.004 |
| Amiloride | 99.02 ± 0.002 | 98.01 ± 0.008 | 99.03 ± 0.002 | 99.01 ± 0.002 | 98.02 ± 0.002 | 98.01 ± 0.004 |
| Cariporide | 98.01 ± 0.001 | 98.02 ± 0.001 | 99.01 ± 0.001 | 98.01 ± 0.001 | 98.00 ± 0.015 | 98.01 ± 0.001 |
| S0859 | 99.02 ± 0.004 | 99.01 ± 0.004 | 98.01 ± 0.003 | 99.03 ± 0.009 | 97.01 ± 0.004 | 96.02 ± 0.004 |
| E4031 | 99.01 ± 0.005 | 99.04 ± 0.008 | 99.01 ± 0.004 | 99.02 ± 0.006 | 99.01 ± 0.006 | 99.02 ± 0.007 |
NP40 (150 mM), NaCl (150 mM), Tris-HCl pH 8 (50 mM), EDTA pH 8 (5 mM), NaF (10 mM), Na$_2$PO$_4$ (10 mM), Na$_3$VO$_4$ (0.4 mM), and protease inhibitor cocktail (cOmplete Mini-Roche, Germany).

The following antibodies were used at the concentration of 5µg per mg of extracted proteins: LEAF Purified anti-human, Clone TS2/16 (BioLegend, California, USA) to immunoprecipitate the β1-integrin; mAb Kv11.1 (MCK Therapeutics, Italy) to immunoprecipitate Kv11.1.1. After overnight incubation, the immuno-complex was captured by adding 30 µl of protein A/G agarose beads for 2 h at 4°C (with rolling agitation). The agarose beads were washed 3 times in ice-cold wash buffer and 3 times in ice cold PBS followed by addition of 2X Laemli buffer (10 µl) and boiled for 5 min at 95°C. Afterwards, SDS-PAGE was performed. After electrophoresis, proteins were transferred onto PVDF membrane (previously activated) in blotting buffer under cold condition for 1 h at 100 V. The PVDF membrane was then blocked with 5% BSA in T-PBS (0.1% tween) solution for 3h at room temperature to cover the unspecific antibody binding sites on the membrane. SDS-PAGE and antibody incubation were performed as previously described (Becchetti et al., 2017). The following antibodies were used: anti-β1-integrin, RM-12, polyclonal rabbit antibody, dilution 1:1,000 (Immunological Science, Italy); anti-Kv11.1, C54 polyclonal rabbit antibody, dilution 1:1,000 (DLV’AL TOSCANA S.R.L., Italy); anti-NHE1 polyclonal rabbit antibody, dilution 1:500 (Novus Biologicals, Colorado, USA) and anti-tubulin, monoclonal mouse, dilution 1:500 (Santa Cruz Biotechnology, Texas, USA). The following day the membrane was washed with T-PBS (0.1% tween) (15 min x 3 times) and appropriate secondary antibody: (i) conjugated with peroxidase enzyme was dissolved in 5% BSA in T-PBS (0.1% tween) (dilution 1:10,000) for at least 45 min and washed (15 min x 3 times), revealing was performed using ECL solution in dark room (anti-C54 primary antibody) and (ii) for all other primary antibodies, IRDYe 800 CW (LI-COR Biosciences, Nebraska, USA) was dissolved in 5% BSA in T-PBS (0.1% tween) (dilution 1:20,000) for at least 45 min and washed (15 min x 3 times) before membrane scanning using LI-COR Odyssey Scanner (Biosciences, Nebraska, USA).

**RESULTS**

**Cell Adhesion Mediated by β1 Integrin Produces an Early pH$_i$ Alkalization in HCT 116 and HT 29 CRC Cells**

HCT 116 and HT 29 cells were incubated in serum-free medium for different times (0–180 min) onto different ECM substrates: Col-I, FN, and VN. Polylysine or no-coated plastic surfaces were used as integrin-independent or “no-adhesion” controls, respectively. At different time points, pH$_i$ was determined by BCECF-AM. Although with slightly different time courses, both cell lines underwent an early pH$_i$ increase between 0 and 90 min (Figures 1A, B). At 90 min of incubation, pH$_i$ was significantly higher in cells seeded onto Col-I and FN, i.e., two substrates recognized by β1 integrins, which are well expressed in both cell lines (Table 2). Indeed, treatment with a β1 integrin blocking antibody (BV/ML, Martin-Padura et al., 1994) (indicated as “anti-β1 Ab” in Figure 1) not only blocked cell adhesion (panel A’ and B’), but also prevented the pH$_i$ increase triggered by cell adhesion onto Col-I. In particular, pH$_i$ increased from 6.7 at time 0 (i.e., before seeding) to 7.2 at 90 min in cells seeded either onto Col-I or FN. In contrast, pH$_i$ remained close to the time 0 value (6.74 ±0.006 for HCT 116 and 6.66±0.014 for HT 29) in CRC cells seeded onto Col-I and treated with the anti-β1 Ab (insets to Figures 1A, B). The pH$_i$ alkalization was much smaller in cells seeded onto VN (in agreement with the very low expression of β3-integrins in both cell lines, see Table 2), or polylysine or no-coating conditions. Subsequently (i.e., after 90 min of incubation), the pH$_i$ observed in cells seeded on Col-I and FN progressively returned to the control value. At 180 min, cells displayed a pH$_i$ around 7.0, irrespective of growing conditions (Figures 1A, B). Such values were maintained for at least 24 h (the complete data set is given in Table 3). We conclude that the β1 integrin-mediated adhesion triggers an early and transient pH$_i$ alkalization from 6.7 to 7.2 in CRC cells.

**The pH$_i$ Variations Triggered by β1 Integrin-Dependent Adhesion in CRC Cells Depend on NHE1 Activation and Are Modulated by Kv11.1 Activity**

To better determine the mechanism of integrin-dependent pH$_i$ increase, we applied blockers of the different pH-regulating transporters expressed in CRC cells (Table 2). In particular, we tested acetazolamide (a wide CA inhibitor), amiloride (an NHE blocker, in particular of NHE1, as well as of ENaC), cariporide (a specific NHE1 inhibitor), and S0859 (an inhibitor of all NBCs). Drugs were used at the concentrations indicated in Materials and Methods, on cells seeded onto Col-I for 90 min, since the beginning of the experiment. Acetazolamide had no effect on pH$_i$ of either cell line, whereas both amiloride and cariporide produced a statistically significant decrease of pH$_i$, which reached values comparable to those detected in cells before seeding (dotted line in Figure 2). The same effect was produced by cariporide. The treatment with S0859 produced a reduction of pH$_i$, although much lower compared to that obtained with amiloride (Figures 2A, B).
These results suggest that the early alkalization triggered by β1 integrin-mediated adhesion is mostly sustained by the activity of the Na+/H+ antiporter NHE1, with a lesser contribution of NBC, and scarce involvement of carbonic anhydrases.

We then tested whether Kv11.1 was involved in the integrin-dependent pH\textsubscript{I} alkalization. To this purpose, cells were treated with the Kv\textsubscript{11.1} blocker E4031, at 40 μM (Masi et al., 2005). After 90 min of cell adhesion on Col-I, both HCT 116 and HT 29 cells treated with E4031 showed pH\textsubscript{I} values significantly more acidic compared to the untreated controls (Figures 2A, B). Hence, the activity of Kv\textsubscript{11.1} appears to control NHE1 activation, after β1 integrin-mediated adhesion. This
TABLE 3 | Complete set of raw pH$_i$ values.

| HCT 116 | 0' | 30' | 90' | 180' | 360' | 1440 |
|---------|----|-----|-----|------|------|------|
| No Coating | 6.70 ± 0.008 | 6.82 ± 0.005 | 6.96 ± 0.045 | 7.00 ± 0.003 | 7.10 ± 0.004 | 7.12 ± 0.013 |
| Polylysine | 6.70 ± 0.008 | 6.75 ± 0.036 | 7.03 ± 0.011 | 7.02 ± 0.012 | 7.12 ± 0.013 | 7.12 ± 0.012 |
| Collagen I | 6.70 ± 0.008 | 7.15 ± 0.009 | 7.28 ± 0.007 | 7.01 ± 0.005 | 7.11 ± 0.008 | 7.01 ± 0.015 |
| Fibronectin | 6.70 ± 0.008 | 7.16 ± 0.023 | 7.29 ± 0.026 | 7.08 ± 0.004 | 7.05 ± 0.014 | 7.18 ± 0.04 |
| Vitronectin | 6.70 ± 0.008 | 6.77 ± 0.011 | 6.99 ± 0.016 | 6.99 ± 0.045 | 7.00 ± 0.025 | 7.10 ± 0.015 |

| HT 29 | 0' | 30' | 90' | 180' | 360' | 1440 |
|-------|----|-----|-----|------|------|------|
| No Coating | 6.65 ± 0.014 | 6.81 ± 0.035 | 7.00 ± 0.021 | 6.99 ± 0.01 | 7.09 ± 0.01 | 7.09 ± 0.01 |
| Polylysine | 6.65 ± 0.014 | 6.84 ± 0.096 | 6.99 ± 0.006 | 7.12 ± 0.02 | 7.16 ± 0.001 | 7.16 ± 0.001 |
| Collagen I | 6.65 ± 0.014 | 7.22 ± 0.003 | 7.21 ± 0.005 | 7.03 ± 0.027 | 7.11 ± 0.023 | 7.11 ± 0.023 |
| Fibronectin | 6.65 ± 0.014 | 7.21 ± 0.042 | 7.28 ± 0.024 | 6.98 ± 0.006 | 7.03 ± 0.028 | 7.03 ± 0.028 |
| Vitronectin | 6.65 ± 0.014 | 6.84 ± 0.037 | 6.98 ± 0.027 | 6.99 ± 0.011 | 7.01 ± 0.017 | 7.01 ± 0.017 |

FIGURE 2 | Effect of Acetazolamide, Amiloride, S0859, Cariporide, E4031, and E4031 plus Cariporide on pH$_i$ in cells seeded on collagen I, 90 min treatment, in HCT 116 and HT 29 cells. pH$_i$ values of HCT 116 are reported in panel (A) and for HT 29 in panel (B). Red line: pH$_i$ value at time zero. Number represent mean ± s.e.m (of three different experiments). *, P < 0.05 and ***, P < 0.001. p value panel (A): ***P < 0.001: Control vs Aml: 1.9e^-05, Control vs Carip: 1.3e^-05, Control vs E4031: 0.0001, Control vs E4031+Carip: 1.8e^-05 Acet vs Carip: 1.7e^-05; *P < 0.05: Control vs S0859: 0.03, Aml vs S0859: 0.02, Carip vs S0859: 0.02; S0859 vs E4031+ Carip: 0.02; p value panel (B): ***P < 0.001: Control vs Aml: 1.5e^-05, Control vs Carip: 1.4e^-05, Acet vs Carip: 1.3e^-05, Control vs E4031: 0.0001 and Control vs E4031+Carip: 1.9e^-05; *P < 0.05: Control vs S0859: 0.04, Acet vs S0859: 0.02, S0859 vs E4031+ Carip: 0.02.
interpretation was supported by the observation that the combined treatment with E4031 and cariporide had no further effect on the pH$_i$ value obtained after NHE1 inhibition by cariporide (Figures 2A, B).

Blockade of Either NHE1 or Kv$_{11.1}$ Inhibits Lateral Motility of CRC Cells

Next, based on the known correlation between pH$_i$ and cell motility, we performed experiments of lateral motility on our CRC cell lines, which were seeded onto Col-I for 90 min, and treated with either E4031, or cariporide, or a combination of both. Both cariporide and E4031 produced a statistically significant reduction of the motility index compared to untreated cells (Figures 3A, B). The combined treatment with E4031 and cariporide only slightly increased the inhibitory effects of the single treatments on the motility index of either cell line. We conclude that both Kv$_{11.1}$ and NHE1 are involved in controlling the β1 integrin-dependent cell motility in CRC cells.

β1-Integrin, Kv$_{11.1}$, and NHE1 Form a Macromolecular Complex

We previously showed that cell adhesion onto β1 integrin-dependent substrates (e.g., FN or Col-I), induces Kv$_{11.1}$ activation, as well as the formation of a macromolecular signaling complex between the channel and β1 integrin on the plasma membrane of HCT 116 cells (Crociani et al., 2013). We thus hypothesized that NHE1 could be also recruited in such complex, which could account for the functional cross-talk between integrin receptors, Kv$_{11.1}$, and NHE1 in CRC cells. Hence, we seeded HCT116 cells on either uncoated or Col-I-coated surfaces for 90 min, and immunoprecipitated the extracted proteins with anti-β1 integrin or anti-Kv$_{11.1}$ antibodies. Blots were then revealed, respectively, with anti-Kv$_{11.1}$ or anti-β1 integrin antibodies, as well as with anti-NHE1 antibodies. We observed that β1-integrin co-immunoprecipitated with both Kv$_{11.1}$ and NHE1 in CRC cells before cell seeding (“pre seeding” in Figure 4), indicating the formation of a β1/Kv$_{11.1}$/NHE1 complex, whose assembly was further promoted by cell adhesion onto Col-I for 90 min (lanes 3 and 4 in Figure 4). On the contrary, in cells seeded onto uncoated surfaces, only a weak co-immunoprecipitation was observed between β1-integrin and Kv$_{11.1}$, and no association was observed with NHE1. We conclude that cell adhesion onto Col-I stimulates the formation of a macromolecular complex between β1-integrin, Kv$_{11.1}$, and NHE1.

DISCUSSION

In the present paper, we provide evidence that, in CRC cells, the β1 integrin-mediated adhesion onto ECM proteins such as Col-I and FN triggers an early and transient pH$_i$ alkalinization, from 6.7 to 7.2. The effect is caused by NHE1 activation and is modulated by the activity of the voltage-dependent K$^+$ channel Kv$_{11.1}$. The transporter and the channel appear to cooperate in sustaining the ECM-induced CRC cell motility. Their action is accompanied by the formation of a macromolecular complex between the β1 integrin, Kv$_{11.1}$ and NHE1.

The rapid β1 integrin-dependent pH$_i$ alkalinization in CRC cells is similar to the one initially reported in bovine capillary endothelial cells (Schwartz et al., 1991), which is also induced by integrin engagement (mainly β1), and sustained by activation of the Na$^+$/H$^+$ exchanger. In our model, we confirmed the NHE1
involvement by showing that ECM-dependent alkalinization was blocked by cariporide.

Following Schwartz’s seminal observation, the pH regulatory role of NHE1 in normal and cancer cells has been receiving increasing attention (Stock and Pedersen, 2017). In particular, in CRC cells, both H⁺ extrusion through NHE1 and HCO₃⁻ influx through NBCe1 give a significant contribution to pH regulation. However, while HCO₃⁻ influx appears to represent a constitutive element of pH regulation, the NHE1-mediated H⁺ efflux may vary, depending on culture conditions, e.g. 2D vs 3D cultures (Hulikova et al., 2011). In CRC cells, we found that the Na⁺/HCO₃⁻ cotransporter, although present, provides only a weak contribution to the integrin-dependent alkalinization in CRC cells. In fact, NHE1 appears to constitute the main molecular device linking the ECM microenvironment to pH regulation.

Numerous mechanisms leading to NHE1 activation have been described in the various cell types in which the transporter is expressed (Orlowski and Grinstein, 2004). Stimuli such as growth factors, peptide hormones etc., which activate receptor tyrosine kinases and G protein-coupled receptors, enhance NHE1 activity, through the involvement of the mitogen-activated, extracellular signal-related kinase (MEK-ERK)-p90sk. The latter phosphorylates NHE1, and enables its binding to the multifunctional scaffolding protein 14-3-3 respectively, which triggers the formation of a macromolecular functional complex on the plasma membrane which comprises the channel and the integrin itself. This occurs preferentially when the channel is in the closed conformation, and leads to the activation of signaling pathways which also involve the scaffold protein 14-3-3, and in turn control different aspects of cancer cell behavior (Becchetti et al., 2012; Crociani et al., 2013; Iorio et al., 2020). In cancers, β₁ integrin-mediated adhesion to FN or Col-I activates Kᵥ11.1, and induces the formation of a macromolecular functional complex which goes on to activate hallmarks of CRC as whole to contribute to trigger a complex signaling pathway which in turn regulates NHE1-dependent motility and invasion in different cancer cells (Cardone et al., 2005). In particular, NHE1 is linked to the actin cytoskeleton and integrates phosphorylation signals arising from kinases which are involved in cytoskeletal reorganization and cell motility. In addition, NHE1 is preferentially localized in pseudopodia, focal adhesion plates, and invadopodia in migrating cells (Paradiso et al., 2004; Patel and Barber, 2005; Clement et al., 2013). In this context, a slight alkalinization mediated by NHE1 was found to regulate the coflin-mediated actin assembly (Frantz et al., 2008), a central mechanism in cell protrusion. Hence, NHE1 and coflin respectively act as a pH regulator and a pH sensor, to mediate actin filament assembly.

The most novel result emerging from our data is that the Kᵥ11.1 channel is implicated in the pH₇₅ alkalinization triggered by integrin-mediated cell adhesion to ECM proteins, and sustained by NHE1 activity. Kᵥ11.1 is over-expressed in many cancer types, including CRC (Lastraioili et al., 2004; Lastraioili et al., 2012; Crociani et al., 2013; Iorio et al., 2020). In cancers, β₁ integrin-mediated adhesion to FN or Col-I activates Kᵥ11.1, and induces the formation of a macromolecular functional complex on the plasma membrane which comprises the channel and the integrin itself. This occurs preferentially when the channel is in the closed conformation, and leads to the activation of signaling pathways, which also involve the scaffold protein 14-3-3, and in turn control different aspects of cancer cell behavior (Becchetti et al., 2017; Becchetti et al., 2019). The recruitment of NHE1 in the Kᵥ11.1/β₁ integrin complex could give rise to the formation of a signaling hub, facilitating NHE1 activation and hence a
localized pH, alkalization, which in turn could affect the reorganization of actin filaments, presumably regulated by coflin activation. This agrees with our recent observations in pancreatic ductal adenocarcinoma cells, where KV11.1 regulates cell migration through a reorganization of f-actin in stress fibers and a modulation of filopodia formation and dynamics (Manoli et al., 2019).

The identification of the signaling mechanisms underlying KV11.1 and NHE1 interaction triggered by β1 integrin–mediated adhesion needs further experiments. Nevertheless, the interplay between a K+ channel and the pH regulating transporter NHE1 that we describe in the present paper can be considered of relevance in the context of CRC invasiveness/motility. This aspect is often dependent on a complex interaction between cancer cells and the tumor microenvironment, and in particular with ECM proteins like collagens and fibronectin (Arcangeli, 2011). Finally, targeting the integrin/ion channel/NHE1 molecular hub might represent a therapeutic option to fight cancer invasiveness.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JI performed the experiments, analyzed the data, prepared the figures, wrote the manuscript. CD performed the experiments and wrote the manuscript. TL performed the experiments. EL helped in the analysis of the data and reviewed the manuscript. GB contributed to experiments and figures. AB reviewed manuscript. AA designed this project and wrote the manuscript.

REFERENCES

Arcangeli, A., Crociani, O., Lastraioli, E., Masi, A., Pillozzi, S., and Becchetti, A. (2009). Targeting ion channels in cancer: a novel frontier in antineoplastic therapy. Curr. Med. Chem. 16, 66–93. doi: 10.2174/092986709787002835
Arcangeli, A. (2011). Ion channels and transporters in cancer. 3. Ion channels in the tumor cell-microenvironment cross talk. Am. J. Physiol. Cell Physiol. 301, C762–C771. doi: 10.1152/ajpcell.00113.2011
Bauer, C. K., and Schwarz, J. R. (2018). Ether-à-go-go (Kv11) potassium channels: effective modulators of neuronal excitability. J. Physiol. 596, 769–783. doi: 10.1113/JP275477
Becchetti, A., Crescioli, S., Zanieri, F., Petroni, G., Mercatelli, R., Coppola, S., et al. (2017). The conformational state of hERG1 channels determines integrin association, downstream signaling, and cancer progression. Sci. Signal. 10, eaaf3236. doi: 10.1126/scisignal.aaf3236
Becchetti, A., Petroni, G., and Arcangeli, A. (2019). Ion Channel Conformations Regulate Integrin- Dependent Signaling. Trends Cell Biol. 4, 298–307. doi: 10.1016/j.tcb.2018.12.005
Benoit, Y. D., Larrivee, J. F., Groulx, J. F., Stankova, J., Vachon, P. H., and Beaulieu, J. F. (2010). Integrin αβ1 confers anoikis susceptibility to human intestinal epithelial crypt cells. Biochem. Biophys. Res. Commun. 399, 434–439. doi: 10.1016/j.bbrc.2010.07.107
Bose, T., Cieslar-Pobuda, A., and Wiechec, E. (2015). Role of ion channels in regulating Ca2+ homeostasis during the interplay between immune and cancer cells. Cell Death Dis. 6, e1648. doi: 10.1038/cddis.2015.23
Boudjidi, S., Carrier, J., Groulx, J., and Beaulieu, J. F. (2016). Integrin αβ1 expression is controlled by c-MYC in colorectal cancer cells. Oncogene 35, 1671–1678. doi: 10.1038/onc.2015.231

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SUPPLEMENTARY MATERIAL

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clinicalopathological correlations and therapeutic implications. Clin. Cancer Res. 20, 1502–1512. doi: 10.1158/1078-0432.CCR-13-2633
Demaures, N., Downey, G. P., Waddell, T. K., and Grinstein, S. (1996). Intracellular pH regulation during spreading of human neutrophils. J. Cell Biol. 133, 1391–1402. doi: 10.1083/jcb.133.6.1391
Eble, J. A., and Tuckwell, D. S. (2003). The alpha2beta1 integrin inhibitor rhodocitin binds to the A-domain of the integrin alpha2 subunit proximal to the collagen-binding site. Biochem. J. 376, 77–85. doi: 10.1042/bj0300737
Frantz, C., Barreiro, G., Dominguez, L., Chen, X., Eddy, R., Condeels, J., et al. (2008). Collin is a pH sensor for actin free barbed end formation: role of phosphoinositide binding. J. Cell Biol. 183, 865–879. doi: 10.1083/jcb.200804161
Gadsby, D. C. (2002). Ion channels versus ion pumps: the principal difference, in Reference to a different article:
Grinstein, S., Woodside, M., Waddell, T. K., Downey, G. P., J Orlowski, J., Pouyssegur, J., et al. (1993). Focal localization of the Na+/H+ antipporter in the etiopathogenesis of colorectal cancer. Nat. Rev. Mol. Cell Biol. 13, 3268–3281. doi: 10.1038/mbc.12.10.3268
Grant, R. L., and Acosta, D. (1997). Ratiometric measurement of intracellular pH. Eur. J. Biochem. 253, 565–572. doi: 10.1111/j.1432-1033.1997.9700106.x
Lastraioli, E., Perrone, G., Sette, A., Fiore, A., Grinstein, S., et al. (2015a). b. hERG1 channels drive tumour malignancy and may serve as prognostic factor in pancreatic ductal adenocarcinoma. Br. J. Cancer 112, 1076–1087. doi: 10.1038/bjc.2015.28
Iorio, E., Arcangeli, A. (2015b). a. Ion channel expression as promising cancer biomarker. Biochim. Biophys. Acta 1848, 2685–2702. doi: 10.1016/j.bbamem.2014.12.016
Iorio et al. Kv11.1 and NHE1 in CRC

Frontiers in Pharmacology | www.frontiersin.org June 2020 | Volume 11 | Article 84810
Pinheiro, C., Longatto-Filho, A., Azevedo-Silva, J., Casal, M., Schmitt, F. C., and Baltazar, F. (2012). Role of monocarboxylate transporters in human cancers: state of the art. *J. Bioenerg. Biomembr.* 44, 127. doi: 10.1007/s10863-012-9428-1

Plopper, G. E., McNamee, H. P., Dike, L. E., Bojanowski, K., and Ingber, D. E. (1995). Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Mol. Biol. Cell.* 6, 1349–1365. doi: 10.1091/mbc.6.10.1349

Putney, L. K., Denker, S. P., and Barber, D. L. (2002). The changing face of the Na+/H+ exchanger, NHE1: structure, regulation, and cellular actions. *Annu. Rev. Pharmacol. Toxicol.* 42, 527–552. doi: 10.1146/annurev.pharmtox.42.092001.143801

Schmidt, R., Streit, M., Kaiser, R., Herzberg, F., Schirner, M., Schramm, K., et al. (1998). *De novo* expression of the α5β1-fibronectin receptor in HT29 colon-cancer cells reduces activity of c-src. Increase of c-src activity by attachment on fibronectin. *Int. J. Cancer* 76, 91–98. doi: 10.1002/(sici)1097-0215(19980330)76:1<91::aid-ijc15>3.0.co;2-j

Schwartz, M. A., Both, G., and Lechene, C. (1989). Effect of cell spreading on cytoplasmic pH in normal and transformed fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 86, 4525–4529. doi: 10.1073/pnas.86.12.4525

Schwartz, M. A., Ingber, D. E., Lawrence, M., Springer, T. A., and Lechene, C. (1991). Share the Ability to Induce Elevation of Intracellular pH. *Exp. Cell Res.* 195, 533–555. doi: 10.1006/0014-4827(91)90407-L

Sennoune, S. R., Luo, D., and Martínez-Zaguilán, R. (2004). Plasmalemmal vacuolar-type H+−ATPase in cancer biology. *Cell Biochem. Biophys.* 40, 185. doi: 10.1385/CBB:40:2:18

Sharma, M., Astekar, M., Soi, S., Manjunatha, B. S., Shetty, D. C., and Radhakrishnan, R. (2015). pH Gradient Reversal: An Emerging Hallmark of Cancers. *Recent Patents Anti-Cancer Drug Discov.* 10, 244–258. doi: 10.2174/157492810666150708110608

Srivastava, J., Barreiro, G., Groscurth, S., Gingras, A. R., Gough, B. T., Critchley, D. R., et al. (2008). Structural model and functional significance of pH-dependent talin–actin binding for focal adhesion remodeling. *J. PNAS* 105, 14436–14441. doi: 10.1073/pnas.0805163105

Stock, C., and Pedersen, S. (2017). Roles of pH and the Na+/H+ exchanger NHE1 in cancer: From cell biology and animal models to an emerging translational perspective? *Semin. Cancer Biol.* 43, 5–16. doi: 10.1016/j.semcancer.2016.12.001

Stock, C., and Schwab, A. (2006). Role of the Na/H exchanger NHE1 in cell migration. *Acta Physiol. (Oxf)* 187, 149–157. doi: 10.1111/j.1748-1716.2006.01543.x

Stock, C., and Schwab, A. (2009). Protons make tumor cells move like clockwork. *Pflugers Arch.* 458, 981–992. doi: 10.1007/s00424-009-0677-8

Stock, C., Gassner, B., Hauck, C. R., Arnold, H., Mally, S., Eble, J. A., et al. (2005). Migration of human melanoma cells depends on extracellular pH and Na+/H+ exchange. *J. Physiol.* 567, 225–238. doi: 10.1113/jphysiol.2005.088344

Stock, C., Cardone, R. A., Busco, G., Krahling, H., Schwab, A., and Reshkin, S. J. (2008). Protons extruded by NHE1: digestive or glue? *Eur. J. Cell Biol.* 87, 591–599. doi: 10.1016/j.ejcb.2008.01.007

Webb, B. A., Chimenti, M., Jacobson, M. P., and Barber, D. L. (2011). Dysregulated pH: a perfect storm for cancer progression. *Nat. Rev. Cancer* 11, 671–677. doi: 10.1038/nrc3110

Yatohgo, T., Izumi, M., Kashiwagi, H., and Hayashi, M. (1988). Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* 13, 281–292. doi: 10.1247/csf.13.281

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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