



# Calcium receptor signaling and citrate transport

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Received: 8 August 2017 / Accepted: 10 January 2018 / Published online: 30 January 2018  
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## Abstract

The calcium sensing receptor (CaSR) in the distal nephron decreases the propensity for calcium stones. Here we investigate if the apical CaSR in the proximal tubule also prevents stone formation acting via regulation of apical dicarboxylate and citrate transport. Urinary citrate, partially reabsorbed as a dicarboxylate in the proximal tubule lumen, inhibits stone formation by complexing calcium. We previously demonstrated a novel apical calcium-sensitive dicarboxylate transport system in OK proximal tubule cells. This calcium-sensitive process has the potential to modulate the amount of citrate available to complex increased urinary calcium. Using isotope labeled succinate uptake in OK cells along with various pharmacologic tools we examined whether the CaSR alters apical dicarboxylate transport and through which signal transduction pathways this occurs. Our results indicate that in the proximal tubule CaSR adjusts apical dicarboxylate transport, and does so via a  $\text{CaSR} \rightarrow \text{G}_q \rightarrow \text{PKC}$  signaling pathway. Thus, the CaSR may decrease the propensity for stone formation via actions in both proximal and distal nephron segments.

**Keywords** CaSR · Calcium-sensitive · Dicarboxylate transport · Citrate · Apical · Proximal tubule

## Introduction

The calcium sensing receptor (CaSR) may play a role in decreasing the propensity for calcium stones. The CaSR is present in many tissues and has many roles. In the kidney, it is located throughout the nephron and has a multitude of functions varying with segment, polarity and cell type [1–4]. Importantly, some of these functions in distal nephron segments are thought to prevent stone formation [5, 6]. Of note in the proximal tubule the CaSR is located on the apical membrane [7] and is found in the proximal tubule opossum kidney (OK) cell model [3, 8]. CaSR has been found to modulate both fluid reabsorption and  $\text{H}^+$  secretion in the proximal tubule and gastric gland, perhaps via increases in intracellular calcium levels and ERK activity [9, 10]. The present study in OK cells explores a mechanism whereby CaSR in the proximal tubule may prevent stone formation acting via apical dicarboxylate (and citrate) transport.

Urinary citrate is the most important endogenous inhibitor of calcium nephrolithiasis. Citrate reabsorption in the upstream nephron determines urinary citrate which is available to complex calcium. The bulk of citrate transport is likely via the proximal tubule apical sodium dicarboxylate cotransporter (NaDC1) which reabsorbs dicarboxylic acids/Krebs cycle intermediates from the glomerular filtrate [11–14]. In previous studies, we used the established OK cell model to study citrate and succinate transport and found inhibition by normal (1.2 mM) levels of extracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_o$ ) [15–18]. (Succinate is used as a test dicarboxylate since its ionic state is not pH sensitive in the normal pH range.) In our prior studies a novel apical calcium-sensitive dicarboxylate transport process was found in OK cells; herein we examine the mechanism of calcium-sensitivity.

Although the initial purpose of the present studies was to determine whether CaSR regulates dicarboxylate/citrate transport, understanding the possible signal transduction mechanisms of this may be important in subsequent efforts to modulate citrate transport and hence stone formation via CaSR. The CaSR is known to couple variously to  $\text{G}_q$ ,  $\text{G}_i$  and  $\text{G}_s$  signaling proteins [19, 20] all of which are found in the proximal tubule [21, 22]. Our aim is to establish if calcium-sensitivity of dicarboxylate transport is due to CaSR activation and what roles  $\text{G}_q$ ,  $\text{G}_i$  and  $\text{G}_s$  play. Signaling via  $\text{G}_q$

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**Table 1** Pharmacological agents, dose and duration

Pharmacological	Dose	Duration	Vehicle	References
Spermine	1 mM	3' during uptake	H <sub>2</sub> O	[25, 26]
Thapsigargin	10 μM	30' prior	DMSO	[27]
PMA	50 nM	30' prior	DMSO	[28, 29]
MDL	50 μM	30' prior	DMSO	[30]
8Br-cAMP	100 μM	30' prior	H <sub>2</sub> O	[31]
MDL + 8Br	50 μM + 100 μM	30' prior	DMSO + H <sub>2</sub> O	[30, 31]
PTX	100 ng/ml	Overnight	H <sub>2</sub> O	[32]
Forskolin	10 μM	30' prior	H <sub>2</sub> O	[30]

The pharmacological agents used in this study, listed in order of appearance are often used to investigate the signaling cascades involved in G-protein coupled receptor activation such as the proximal tubule apical CaSR shown in Fig. 1. Spermine is a known orthosteric CaSR activator and was used in both transport and cell assay experiments [25, 26]. Thapsigargin (Thaps) is commonly used to increase intracellular Ca<sup>2+</sup> [27]. Phorbol 12-myristate 13-acetate (PMA) is a protein kinase C (PKC) activator [28, 29]. MDL-12,330A (MDL) is an adenylate cyclase (AC) inhibitor [30]. 8-Br-cAMP (8Br) is a cell membrane permeable form of cAMP [31]. The combination MDL + 8Br is used to examine the effects of increases of intracellular cAMP without the addition of effects from the upstream components of the signaling cascade. Pertussis toxin (PTX) is used to directly inhibit signaling by G<sub>i</sub> protein [32]. Forskolin is a known AC activator and is used to increase endogenous cAMP [30].

activates phospholipase C (PLC) resulting in the release of [Ca<sup>2+</sup>]<sub>i</sub> stores and activation of PKC [23]. G<sub>i</sub> and G<sub>s</sub> inhibit and activate adenylate cyclase (AC) respectively [19, 24].

## Methods

As previously described, OK cells were maintained in MEM containing 26 mM HCO<sub>3</sub><sup>-</sup> supplemented with 10% FCS (Invitrogen), 25 mM HEPES, 11 mM L-glutamine and 100 IU/ml penicillin in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C [15–17]. Cell monolayers were grown on 24-well plates (Corning-Costar), with media changes every 2 days. After reaching confluence, cell monolayers were changed to serum-free media for a minimum of 24 h before experiments were performed.

Dicarboxylate transport was measured by uptake of radiolabeled [<sup>14</sup>C]-succinate into cell monolayers. Just prior to uptake, cells were rinsed free of media and equilibrated for 2 min at 37 °C in buffer containing either normal (1.2 mM) or low (< 60 μM) [Ca<sup>2+</sup>]<sub>o</sub>. The remaining components of the buffer were as follows (in mM): 109 NaCl, 3 KCl, 2 KH<sub>2</sub>PO<sub>4</sub>, 1 MgSO<sub>4</sub>, 5 alanine, 8.3 glucose, 1 sodium acetate, 25 HEPES; osmolality of 290 mosmol/kg H<sub>2</sub>O and pH 7.4.

Uptake was performed at 37 °C and started by adding 0.4 ml of uptake buffer with 0.626 mCi/ml [1,4-<sup>14</sup>C]-succinate (Moravek Biochemicals) to individual wells. Uptake buffer also contained [<sup>3</sup>H] mannitol (Perkin Elmer) to determine the residual extracellular volume. After 3 min, buffer was removed and wells were rinsed 3X with ice cold 0.1 M MgCl<sub>2</sub>; monolayers were lysed with 1 ml of 0.1 N NaOH and transferred to vials for liquid scintillation counting [15–18].

Uptake was calculated from measured <sup>14</sup>C radioactivity per well; appropriate windows and crossover calculations were used to distinguish between <sup>3</sup>H and <sup>14</sup>C. Uptake was further factored for residual extracellular volume, not removed by triplicate rinsing, calculated from the residual [<sup>3</sup>H] mannitol. Because of variability in absolute transport rates, each experiment was performed in a paired fashion with both normal and low [Ca<sup>2+</sup>]<sub>o</sub> solutions, with a minimum of four separate passages of OK cells for each experiment. Data are expressed as means ± SE. All statistical analysis was performed using one-way ANOVA. Statistical significance was defined as *p* < 0.05.

Unless otherwise stated all pharmacologicals were purchased through Sigma Aldrich (St. Louis, MO). Table 1 lists pharmacological agents, vehicle, dose, duration and references.

A series of colorimetric ELISA cAMP EIA kit competitive binding assays (Cayman Chemical, 581001) were performed on 96 well plates, with standard curves in duplicate and samples in triplicate. As above OK cells were grown on 24 well plates. Buffers containing either normal or low [Ca<sup>2+</sup>]<sub>o</sub> were incubated at 37 °C. MgSO<sub>4</sub> was not included in low [Ca<sup>2+</sup>]<sub>o</sub> solutions as Mg<sup>2+</sup> is a CaSR activator and in low [Ca<sup>2+</sup>]<sub>o</sub> buffer Mg<sup>2+</sup> may be more likely to interfere. All other buffers and pharmacologicals were the same used in uptake experiments. Cells were treated with appropriate buffers and pharmacologicals, buffers were aspirated, 54 μl of 0.1 M HCl was added to each 1.9 cm<sup>2</sup> well, and incubated for 20 min at RT. Cells were scraped off, dissociated; lysates were centrifuged at 1000×*g* for 10 min followed by collection of supernatants. Standard curves and samples were prepared as per kit directions. Samples were diluted 2X and read at 412 nM.

Data are expressed as means  $\pm$  SE. Each mean is derived from four individual experiments studied on 4 separate days. One-way ANOVA was used for statistical comparison in cAMP assays. Statistical significance was defined as  $p < 0.05$ .

## Results

Our previous studies determined that normal levels (1.2 mM) of apical  $[Ca^{2+}]_o$  inhibit apical dicarboxylate transport in OK cell monolayers [15–17]. CaSR is activated only when  $[Ca^{2+}]_o$  is in the range of 0.5–2 mM  $[Ca^{2+}]_o$  [8]. Since the CaSR is at the apical membrane of the proximal tubule, CaSR activation may be the basis of the apical calcium-sensitive dicarboxylate transport we previously reported [15–18]. If CaSR regulates apical dicarboxylate transport due to changes in luminal  $[Ca^{2+}]_o$  levels, then apical addition of CaSR agonists may be used to test this hypothesis.

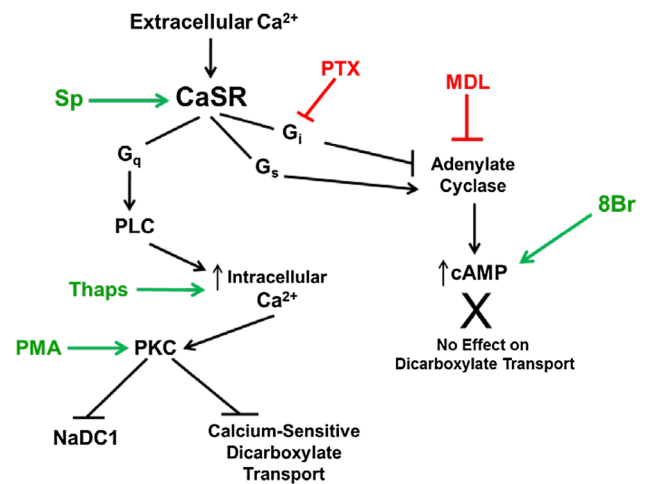
### CaSR activation

To assess if CaSR activation is involved in alterations in apical dicarboxylate transport with changes in luminal  $Ca^{2+}$  OK cell monolayers were treated with 1 mM spermine for 3 min during uptake (Table 1). Spermine an orthosteric activator of CaSR [25, 26] directly activates CaSR without requiring the presence of  $Ca^{2+}$  [25]. Spermine activation of CaSR resulted in the inhibition of dicarboxylate transport in both normal and low  $[Ca^{2+}]_o$  (Fig. 2). As in our previous studies, low  $[Ca^{2+}]_o$  (vehicle control) transport was increased ( $174 \pm 29.8\%$  of control) compared to normal  $[Ca^{2+}]_o$  (vehicle control) at 100%. However, CaSR activation by spermine reduced transport both in normal  $[Ca^{2+}]_o$  ( $100 \pm 0.0$  vs  $71.6 \pm 1.7\%$  of control,  $p < 0.05$ ) and low  $[Ca^{2+}]_o$  ( $174 \pm 29.8$  vs  $123 \pm 25.3\%$  of control,  $p < 0.01$ ). Thus, activation of CaSR reduced dicarboxylate transport at both levels of  $[Ca^{2+}]_o$  demonstrating the contribution of CaSR in apical dicarboxylate transport regulation.

Shown in Fig. 2 the addition of spermine in normal  $[Ca^{2+}]_o$  resulted in additional inhibition of dicarboxylate transport. The CaSR is generally inactive at  $[Ca^{2+}]_o$  levels below 0.2 mM with activation threshold that can vary from 0.5 to 2 mM depending on cell type and levels of expression [8]. This additional level of inhibition may be an additive effect in which spermine, an orthosteric activator, increases CaSR activity beyond that which is observed in normal  $[Ca^{2+}]_o$ .

### $G_q$ signaling lowers dicarboxylate transport

Next we sought to determine which G-protein pathway the CaSR signals via in regulation of apical dicarboxylate/

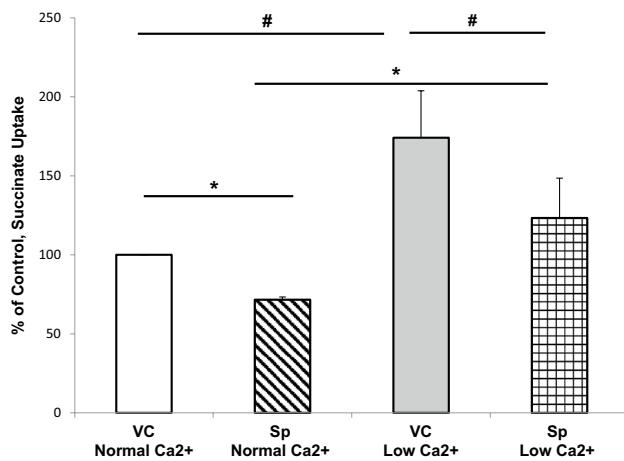


**Fig. 1** Proximal tubule apical CaSR signaling cascade. The apical proximal tubule CaSR potentially signals via the G-proteins:  $G_q$ ,  $G_i$  or  $G_s$  in OK cell monolayers. Figure illustrates an overview of CaSR signaling, the G-protein signaling pathways studied and pharmacologicals used to determine the role of extracellular  $Ca^{2+}$  in apical dicarboxylate transport regulation in OK proximal tubule cell monolayers. Time of treatment, concentration and vehicle controls are outlined in Table 1. Green arrows indicate stimulation, while red bars indicate inhibition by the pharmacological agents used in this study. Black arrows, up arrows, represent stimulation or increases and bars, perpendicular symbols, inhibition of some of the major components of G-protein coupled signaling cascade

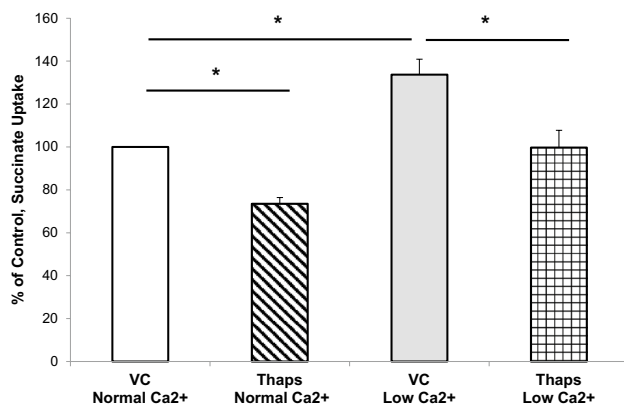
citrate transport. As shown in Fig. 1,  $G_q$  signaling is known to increase  $[Ca^{2+}]_i$  via PLC followed by PKC activation. If CaSR activation regulates dicarboxylate transport via  $G_q$  signaling, raising  $[Ca^{2+}]_i$  may alter transport rates. OK cells were pretreated with thapsigargin for 30 min prior to uptake measurements to increase  $[Ca^{2+}]_i$ . Signaling via  $G_q$  activates PLC resulting in increased intracellular  $Ca^{2+}$  and PKC activation (Fig. 1) [33]. In HEK293 cells transfected with human CaSR, PLC activation followed by PKC activation is a  $G_q$ -dependent and  $G_i$ -independent process [34]. Thapsigargin is often used to increase  $[Ca^{2+}]_i$  [27].

Thapsigargin (Fig. 3) decreased dicarboxylate transport in both normal ( $100 \pm 0.0$  vs  $73.5 \pm 2.9\%$  of control,  $p < 0.01$ ) and low  $[Ca^{2+}]_o$  ( $134 \pm 7.2$  vs  $100 \pm 8.2\%$  of control,  $p < 0.01$ ). This indicates that CaSR activation followed by increases in  $[Ca^{2+}]_i$  via  $G_q$  signaling results in decreased dicarboxylate transport in both normal and low  $[Ca^{2+}]_o$ .

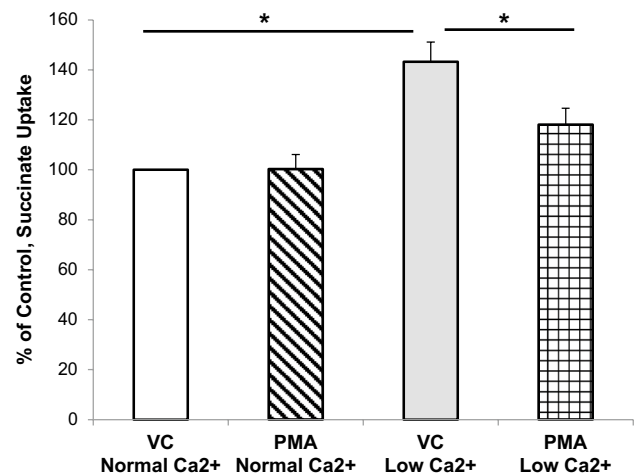
Similar to the dicarboxylate transport processes we are currently studying, BGT1, the renal betaine/GABA transporter is also inhibited by  $[Ca^{2+}]_o$  [35]. Figure 3 shows that thapsigargin treatment resulted in dicarboxylate transport inhibition in both normal and low  $[Ca^{2+}]_o$ . This plus the spermine results (Fig. 2) provides evidence that the CaSR via the  $G_q$  pathway regulates dicarboxylate transport and  $G_q$  signaling increases  $[Ca^{2+}]_i$  possibly followed by PKC activation.



**Fig. 2** Apical CaSR activation results in inhibition of apical dicarboxylate transport. We have shown in our previously published studies [16–18] and herein that decreasing extracellular Ca<sup>2+</sup>, [Ca<sup>2+</sup>]<sub>o</sub>, results in augmentation of apical dicarboxylate transport (compare bar 3 from left with bar 1) in the OK proximal tubule cell line. Succinate transport increased to 174 ± 29.8% of control in low [Ca<sup>2+</sup>]<sub>o</sub> compared to normal [Ca<sup>2+</sup>]<sub>o</sub>. Addition of spermine (1 mM, Sp) to activate the apical CaSR resulted in decreasing succinate transport in both normal (bar 2) and low [Ca<sup>2+</sup>]<sub>o</sub> (bar 4). Spermine addition decreased succinate transport in normal [Ca<sup>2+</sup>]<sub>o</sub> (bar 2) to 71.6 ± 1.7% of control, *p* < 0.05. In low [Ca<sup>2+</sup>]<sub>o</sub> (bar 3, VC) succinate transport was 174 ± 29.8% of that in normal [Ca<sup>2+</sup>]<sub>o</sub>; addition of spermine to low [Ca<sup>2+</sup>]<sub>o</sub> reduced dicarboxylate transport to 123 ± 25.3% of control, *p* < 0.01 (bar 4). Taken together the addition of spermine to OK cell monolayers results in decreased dicarboxylate transport indicating the CaSR is involved in the regulation of apical dicarboxylate transport in the proximal tubule. #*p* < 0.05, \**p* < 0.01, VC vehicle control



**Fig. 3** G<sub>q</sub> signaling is involved in dicarboxylate transport regulation in both normal and low [Ca<sup>2+</sup>]<sub>o</sub>. Activation of apical CaSR may result in signaling via one of 3 G-proteins as illustrated in Fig. 1. This series of experiments looks at G<sub>q</sub> signaling and used thapsigargin (Thaps) pretreatment (10 μM, 30 min) to increase intracellular calcium [Ca<sup>2+</sup>]<sub>i</sub> and thus mimic signaling via G<sub>q</sub>. Thaps pretreatment resulted in decreased dicarboxylate transport in both normal (100 ± 0.0 vs 73.5 ± 2.9% of control, *p* < 0.01) and low [Ca<sup>2+</sup>]<sub>o</sub> (134 ± 7.2 vs 100 ± 8.2% of control, *p* < 0.01). \**p* < 0.01. VC vehicle control. This indicates that following CaSR activation by calcium, G<sub>q</sub> signaling is involved in apical dicarboxylate regulation



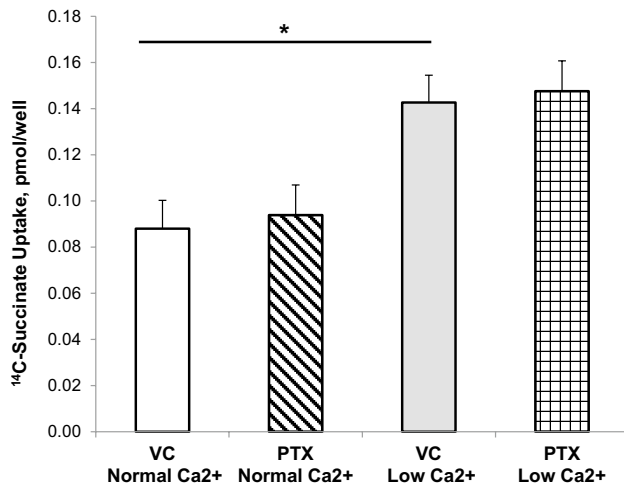
**Fig. 4** PKC activation. G<sub>q</sub> signaling produces increases in intracellular Ca<sup>2+</sup> and is known to activate PKC. Since increasing [Ca<sup>2+</sup>]<sub>i</sub> with Thaps shown in Fig. 3 is involved; our next step was to directly activate PKC and examine changes in transport. OK cell monolayers were pretreated with phorbol 12-myristate 13-acetate (50 nM PMA) for 30 min to activate endogenous PKC. Succinate transport in normal [Ca<sup>2+</sup>]<sub>o</sub> was unchanged (100 ± 0.0 vs 100 ± 5.9% of control, *p* = NS). However, PMA addition resulted in decreased dicarboxylate transport in low [Ca<sup>2+</sup>]<sub>o</sub> (143 ± 7.8 vs 118 ± 6.6% of control, *p* < 0.01). Thus, when changes/decreases in [Ca<sup>2+</sup>]<sub>o</sub> occur, CaSR signaling via G<sub>q</sub> → PKC is involved in apical dicarboxylate transport regulation. Thus, PKC activation is likely part of the signaling cascade (Fig. 1) that results in lower levels of apical dicarboxylate transport in normal concentrations of [Ca<sup>2+</sup>]<sub>o</sub>. VC vehicle control. \**p* < 0.01

### PKC activation decreases dicarboxylate transport

The results described above show that both spermine and thapsigargin addition in low extracellular Ca<sup>2+</sup> mimics transport inhibition in normal [Ca<sup>2+</sup>]<sub>o</sub> and thus links the CaSR to apical dicarboxylate transport regulation. G<sub>q</sub> signaling increases [Ca<sup>2+</sup>]<sub>i</sub> followed by PKC activation; and PKC is known to regulate many cell membrane transporters [28, 29, 35–37]. PKC has also been shown to affect NaDC1 [38]. To investigate if PKC activation regulates apical dicarboxylate transport via G<sub>q</sub>, OK cell monolayers were pretreated with the PKC activator, 50 nM phorbol 12-myristate 13-acetate (PMA) for 30 min. Shown in Fig. 4, succinate transport in normal [Ca<sup>2+</sup>]<sub>o</sub> was unchanged (100 ± 0.0 vs 100 ± 5.9% of control, *p* = NS). However, in low [Ca<sup>2+</sup>]<sub>o</sub> PMA inhibited dicarboxylate transport (143 ± 7.8 vs 118 ± 6.6% of control, *p* < 0.01) and reveals that signaling via PKC is involved in apical dicarboxylate transport regulation.

### G<sub>i</sub> inhibition

Thus far using spermine to activate CaSR, thapsigargin to increase [Ca<sup>2+</sup>]<sub>i</sub> (intracellular calcium), and PMA to activate PKC have demonstrated that dicarboxylate transport regulation likely occurs through CaSR → G<sub>q</sub> → PKC signaling at



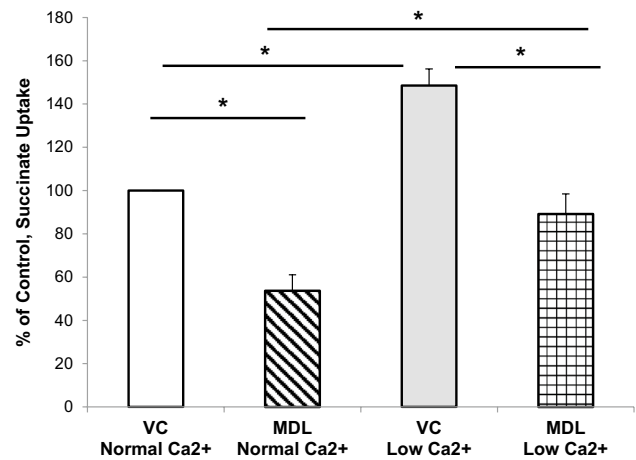
**Fig. 5** Direct inhibition of  $G_i$  protein with PTX does not alter dicarboxylate transport. Possible CaSR signaling via the  $G_i$  protein can be investigated using pertussis toxin (PTX) to directly inhibit  $G_i$ . Signaling via  $G_i$  (Fig. 1) results in the inhibition of adenylate cyclase (AC). PTX, used to block the inhibition of AC, should result in an increase of intracellular cAMP. OK cell monolayers were treated overnight with PTX (100 ng/ml) to inhibit  $G_i$ . Shown here PTX pretreatment did not alter dicarboxylate transport in either normal ( $100 \pm 0.0$  vs  $107 \pm 1.9\%$  of control,  $p = \text{NS}$ ) or low  $[\text{Ca}^{2+}]_o$  ( $174 \pm 24$  vs  $178 \pm 15\%$  of control,  $p = \text{NS}$ ). VC vehicle control.  $*p < 0.01$

normal  $[\text{Ca}^{2+}]_o$ . However, in the proximal tubule the CaSR may also signal via  $G_i$  and  $G_s$ . In some cells, CaSR activation is known to signal via  $G_i$  or  $G_s$  pathways which produce changes in cAMP levels via AC inhibition or activation, respectively [19, 20, 24].

If CaSR activation regulates dicarboxylate transport via  $G_i$  signaling, the direct inhibition of  $G_i$  would be expected to increase dicarboxylate transport in normal  $[\text{Ca}^{2+}]_o$ . To investigate this we used the direct  $G_i$  inhibitor pertussis toxin (PTX). OK cell monolayers were pretreated with PTX 100 ng/ml overnight. Shown in Fig. 5, PTX had little effect on dicarboxylate transport in both normal ( $100 \pm 0.0$  vs  $107 \pm 1.9\%$  of control,  $p = \text{NS}$ ) and low  $[\text{Ca}^{2+}]_o$  ( $174 \pm 24$  vs  $178 \pm 15\%$  of control,  $p = \text{NS}$ ).

### $G_i$ activation

$G_i$  activation inhibits AC and hence cAMP production [19]. To determine if direct pharmacological inhibition of AC results in decreased dicarboxylate transport the AC inhibitor MDL-12, 330A (MDL) 50 nM for 30 min was applied to OK cell monolayers to mimic  $G_i$  activation. If  $G_i$  signaling regulates apical dicarboxylate transport via AC inhibition, MDL should inhibit apical dicarboxylate transport only in low  $[\text{Ca}^{2+}]_o$ . Shown in Fig. 6, MDL inhibited succinate transport in both normal ( $100 \pm 0.00$  vs  $53.7 \pm 7.4\%$  of



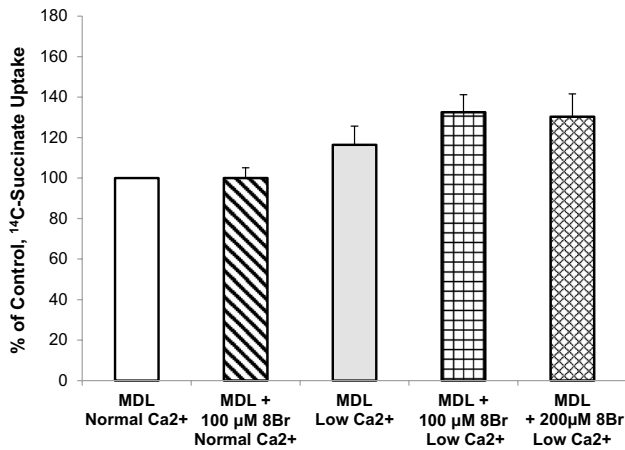
**Fig. 6** Direct inhibition of AC with MDL decreased dicarboxylate transport in both normal and low  $[\text{Ca}^{2+}]_o$ . Another way to investigate  $G_i$  signaling is to directly inhibit AC by the addition of MDL-12,330A (MDL). OK cell monolayers were treated with 50  $\mu\text{M}$  of MDL for 30 min before beginning transport measurements. MDL inhibited succinate transport in both normal ( $100 \pm 0.00$  vs  $53.7 \pm 7.4\%$  of control,  $p < 0.01$ ) and low  $[\text{Ca}^{2+}]_o$  ( $146 \pm 6.4$  vs  $88.7 \pm 9.0\%$  of control,  $p < 0.01$ ). VC vehicle control.  $*p < 0.01$ . The MDL results differ from the results in Fig. 5, where PTX had no effect on dicarboxylate transport; this discrepancy is addressed in the text

control,  $p < 0.01$ ) and low  $[\text{Ca}^{2+}]_o$  ( $146 \pm 6.4$  vs  $88.7 \pm 9.0\%$  of control,  $p < 0.01$ ).

These results with MDL (Fig. 6) are unexpected when compared with those of PTX (Fig. 5). MDL was used to directly inhibit AC (i.e., mimic  $G_i$ ) which resulted in reduced dicarboxylate transport in both normal and low  $[\text{Ca}^{2+}]_o$ . This decrease would be expected if CaSR regulation of dicarboxylate transport was via  $G_i$  signaling. It is possible, however, MDL may decrease dicarboxylate transport in some way other than AC inhibition [39–41]. Some previous studies indicate MDL may have effects unrelated to AC inhibition such as blocking of L-type  $\text{Ca}^{2+}$  channels, altering glycine transport and inhibiting phosphodiesterases [42–44].

### $G_s$ signaling

If  $G_i$  signaling results in dicarboxylate transport regulation by reducing cAMP levels from inhibition of AC (consistent with MDL experiments), then increasing intracellular cAMP by addition of the membrane permeable 8-Br-cAMP (8Br) should result in increased dicarboxylate transport (Fig. 1). Signaling via  $G_s$  is known to result in AC activation which in turn increases intracellular cAMP [24]. In contrast to the MDL experiments, if CaSR activity results in decreased dicarboxylate transport in normal  $[\text{Ca}^{2+}]_o$  due to  $G_s$  activation of AC, increasing intracellular cAMP via 8Br would be expected to decrease dicarboxylate transport.



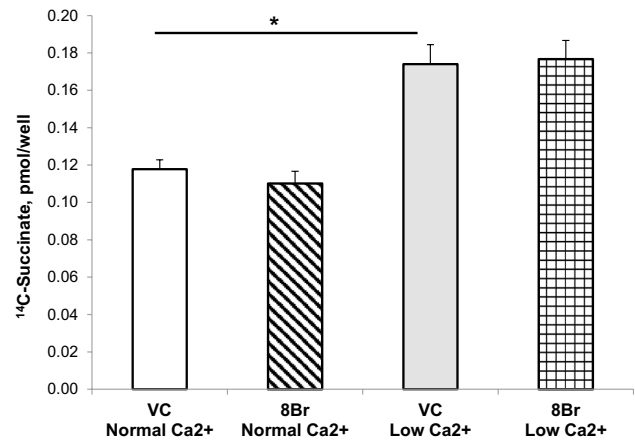
**Fig. 7** Increasing intracellular cAMP does not offset the effects of AC inhibition on dicarboxylate transport. In this series of experiments MDL was used as a direct AC inhibitor to block increases in endogenously generated intracellular cAMP in all four groups of experiments. 8-Br-cAMP (8Br, 100 μM) is a cell membrane permeable form of cAMP [31] and was used to increase intracellular cAMP exogenously. Comparing bars 1 vs 2, increasing intracellular cAMP by the addition of 8Br did not overcome the direct inhibition of AC by MDL in normal  $[Ca^{2+}]_o$  ( $100 \pm 0.00$  vs  $100 \pm 5.1\%$  of control). Similar results were found in low  $[Ca^{2+}]_o$ ; 8Br had no significant effect in the presence of MDL in low  $[Ca^{2+}]_o$  ( $116 \pm 9.2$  vs  $133 \pm 8.7\%$  of control) shown in bars 3 vs 4. And when 8Br concentrations were doubled to 200 μM, again there was no significant effect ( $116 \pm 9.2$  vs  $130 \pm 11.3\%$  of control) shown in bars 3 vs 5. This provides further evidence that inhibition of dicarboxylate transport in both normal and low  $[Ca^{2+}]_o$  (Fig. 6) is not via AC inhibition, indicating that MDL may be acting via another mechanism [39–41]

However, addition of 8Br + MDL (Fig. 7) did not offset the effects of direct AC inhibition via MDL alone (Fig. 6) in normal ( $100 \pm 0.0$  vs  $100 \pm 5.1\%$  of control) or low  $[Ca^{2+}]_o$  ( $116 \pm 9.2$  vs  $133 \pm 8.7\%$  of control) indicating that MDL may be acting via another mechanism [39–41].

### Increasing intracellular cAMP

Because of these varying results, further studies on  $G_i$  signaling were needed to resolve the contrasting evidence with PTX and MDL. If CaSR activation results in decreased dicarboxylate transport via  $G_i$  inhibition of AC, 8Br would be expected to reverse this inhibition and increase dicarboxylate transport due to rises in intracellular cAMP. However, if CaSR activation decreases transport via  $G_s$  resulting in AC activation, 8Br should decrease dicarboxylate transport.

OK cells were pretreated with 100 μM 8Br for 30 min prior to uptake. 8Br (Fig. 8) had no effect on dicarboxylate transport in either normal ( $100 \pm 0.00$  vs  $94.7 \pm 1.7\%$  of control,  $p = NS$ ) or low  $[Ca^{2+}]_o$  ( $154 \pm 9.9$  vs  $131 \pm 9.6\%$  of control,  $p = NS$ ). Thus, neither  $G_i$  nor  $G_s$  are involved in apical dicarboxylate transport regulation. Importantly, these results align with those of PTX (Fig. 5), where inhibition of



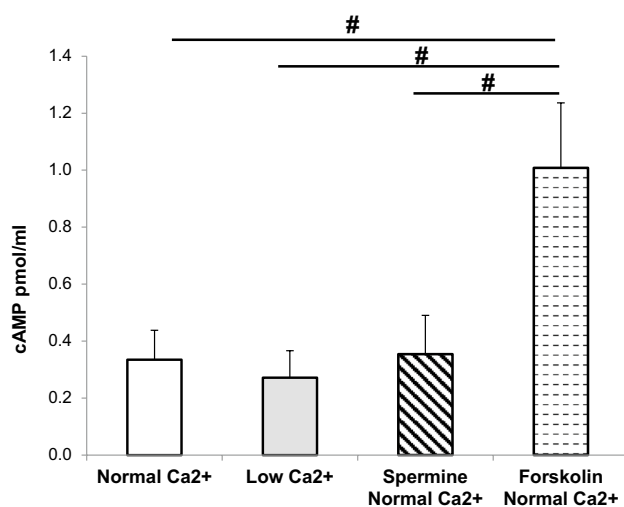
**Fig. 8** Increasing intracellular cAMP via 8-Br-cAMP has no effect on dicarboxylate transport. Intracellular cAMP in OK cell monolayers was increased by the addition of 100 μM 8-Br-cAMP (8Br) for 30 min before start of transport measurements. Raising intracellular cAMP had no effect on dicarboxylate transport in either normal ( $100 \pm 0.00$  vs  $94.7 \pm 1.7\%$  of control,  $p = NS$ ) or low ( $154 \pm 9.9$  vs  $131 \pm 9.6\%$  of control,  $p = NS$ )  $[Ca^{2+}]_o$ . VC vehicle control.  $*p < 0.01$ . This suggests that CaSR activation is not regulating dicarboxylate transport via  $G_s$ . Importantly, these results align with those in Figs. 5 and 7, indicating that neither  $G_i$  nor  $G_s$  are involved in regulation of dicarboxylate transport via CaSR activation

$G_i$  had no effect on dicarboxylate transport and thus supports actions of MDL are not likely the result of inhibiting AC, but some other mechanism not involved with  $G_i$  [39–41].

### cAMP assays

Thus, far the CaSR- $G_q$  signaling studies indicate that CaSR inhibits apical dicarboxylate transport in normal  $[Ca^{2+}]_o$  via  $G_q$ -PKC signaling, but not via  $G_i$  or  $G_s$  signaling. To verify this, intracellular cAMP assays were performed. Endogenously  $G_i$  is an inhibitor of AC and  $G_s$  is an activator. If the CaSR signals via  $G_i$  in OK cells AC inhibition would reduce cAMP levels; however, signaling via  $G_s$  would result in AC activation and increased cAMP levels. Because the CaSR is known for ligand biased signaling (discussed below) it is important to examine the effects of  $[Ca^{2+}]_o$  and spermine on endogenous cAMP levels to determine if there is a signaling bias.

Shown in Fig. 9 the level of  $[Ca^{2+}]_o$  had no effect on intracellular cAMP levels ( $0.335 \pm 0.103$  vs  $0.272 \pm 0.095$  pmol/ml,  $p = NS$ ). This further indicates that activation of the CaSR in OK cells does not signal via  $G_i$  or  $G_s$ . CaSR activator spermine also had little effect on cAMP levels in normal  $[Ca^{2+}]_o$  compared to normal  $[Ca^{2+}]_o$  alone ( $0.335 \pm 0.103$  vs  $0.354 \pm 0.136$  pmol/ml,  $p = NS$ ). Thus both spermine and  $[Ca^{2+}]_o$  likely activate the same CaSR signaling pathway. Forskolin, an AC activator, was used as a control to increase intracellular cAMP. As was expected, forskolin in



**Fig. 9** Assay of Intracellular cAMP Levels. To further examine if there was a role of cAMP in dicarboxylate transport regulation, intracellular levels of cAMP were measured. Shown here, no changes in intracellular cAMP levels were found in low  $[Ca^{2+}]_o$  ( $0.335 \pm 0.103$  vs  $0.272 \pm 0.095$  pmol/ml,  $p=NS$ ). The CaSR activator spermine (1 mM, 5 min) also had no significant effect on intracellular cAMP levels in normal  $[Ca^{2+}]_o$  compared to normal control ( $0.335 \pm 0.103$  vs  $0.354 \pm 0.136$  pmol/ml,  $p=NS$ ). This indicates that CaSR activation in OK cell monolayers does not result in AC activation or signal via  $G_i$  or  $G_s$  which act via changes in intracellular cAMP. As a positive control for the cAMP assays, the AC activator forskolin (10  $\mu$ M, 30 min) significantly increased endogenous intracellular cAMP in normal  $[Ca^{2+}]_o$  ( $0.335 \pm 0.103$  vs  $1.008 \pm 0.228$  pmol/ml,  $p < 0.05$ ). These results further indicate that activation of the CaSR to regulate dicarboxylate transport in OK cells is not signaling via  $G_i$  or  $G_s$ . # $p < 0.05$

normal  $[Ca^{2+}]_o$  resulted in a large increase in cAMP levels compared to normal  $[Ca^{2+}]_o$  alone ( $0.335 \pm 0.103$  vs  $1.008 \pm 0.228$  pmol/ml,  $p < 0.05$ ).

If CaSR activation resulted in  $G_i$  signaling, there should be a reduction in intracellular cAMP levels. If signaling is via  $G_s$  an increase in intracellular cAMP should result since  $G_i$  and  $G_s$  inhibit and activate AC respectively. Figure 9 shows there was little change in cAMP levels when comparing normal and low  $[Ca^{2+}]_o$  indicating that  $[Ca^{2+}]_o$  is not signaling via  $G_i$  or  $G_s$ . The CaSR activator spermine also had no effect on cAMP levels. This along with the PTX and 8Br studies is sufficient evidence to conclude that CaSR activation in OK cells does not result in  $G_i$  or  $G_s$  signaling.

The results with PTX and 8Br on transport, and the measurements of cAMP, indicate that changes in intracellular cAMP are not involved in transducing the CaSR signal involving apical dicarboxylate transport. MDL may be acting via effects unrelated to AC inhibition, explaining the sole discrepant results. MDL has been found to block certain

$Ca^{2+}$  channels, alter glycine transport and inhibit non-cAMP phosphodiesterases [42–44].<sup>1</sup>

Therefore, in OK proximal tubule cells CaSR activation results in  $G_q$  signaling and not  $G_i$  or  $G_s$ . Normal  $[Ca^{2+}]_o$  via activation of the apical CaSR inhibits dicarboxylate transport; spermine, thapsigargin and PMA mimic this regulation in low  $[Ca^{2+}]_o$ . Thus, dicarboxylate transport inhibition occurs via the CaSR  $\rightarrow G_q \rightarrow PKC$  pathway.

## Discussion

Proximal tubule apical dicarboxylate transport is important from several perspectives, but for considerations here as the main regulator of citrate excretion, a potent inhibitor of nephrolithiasis. Citrate concentration in the urine is determined by proximal tubule reabsorption [45, 46]. Dicarboxylate transporters also reabsorb other filtered Krebs cycle intermediates and their downstream delivery (e.g., succinate and  $\alpha$ -ketoglutarate) may be important in downstream receptor signaling which may also impact stone formation [47].

In regards to citrate and stone prevention, teleologically increased urinary or luminal calcium should be matched by increased urinary or luminal citrate to prevent calcium precipitation with anions such as phosphate or oxalate, the complexes of which are not very soluble. Decreased reabsorption of citrate with increased luminal  $Ca^{2+}$  would help achieve this. Our previous studies have established calcium-sensitive dicarboxylate transport at the apical membrane of the OK cell model of the proximal tubule. This calcium-sensitivity may play an important role in preventing stone formation by inhibiting citrate reabsorption in response to increased luminal  $Ca^{2+}$  concentration [15–17]. Thus in the apical membrane of the proximal tubule CaSR activation may serve this function and regulate dicarboxylate transport [3, 7, 48]. Importantly (Fig. 2) the addition of the CaSR activator spermine in low  $[Ca^{2+}]_o$  resulted in inhibition of dicarboxylate transport similar to that observed by increasing extracellular calcium from low to normal  $[Ca^{2+}]_o$ . This demonstrates that CaSR activation regulates apical dicarboxylate transport.

The CaSR prevents  $Ca^{2+}$  stone formation via segment specific functions throughout the nephron. For example, in the distal convoluted and connecting tubules the CaSR and the calcium-selective transient potential vanilloid-subtype 5 channel (TRPV5) co-localize at the luminal membrane and activation of the CaSR results in TRPV5-mediated  $Ca^{2+}$  influx [5]. In the collecting duct,

<sup>1</sup> Complicating this interpretation, NaDC1 has been considered responsible for all citrate reabsorption on the apical side of the proximal tube. We have previously shown that NaDC1 ex vivo is not calcium-sensitive. So whether the calcium sensitive transport is NaDC1 or not remains unknown.

CaSR activation prevents nephrolithiasis by promoting H<sup>+</sup>-ATPase-mediated H<sup>+</sup> excretion and downregulation of Aquaporin 2. This results in urinary acidification and polyuria [6]. Clearly mechanisms in these other nephron segments prevent stone formation via CaSR activation. However, in the proximal tubule such mechanisms have not been described. The present results provide evidence that a mechanism in the proximal tubule may be that calcium activates CaSR which decreases citrate reabsorption, raising urinary citrate and decreasing the propensity for calcium stone formation.

The CaSR in the proximal tubule has been associated with the G<sub>q</sub>, G<sub>i</sub> or G<sub>s</sub> proteins. The G-protein through which it signals depends upon CaSR conformation [25, 49, 50]. Knowing the particular signaling pathway may be useful in the future to modulate citrate excretion. The CaSR has been extensively investigated including its propensity for ligand biased signaling [23, 33, 34, 51–53]. Traditionally there was a two-state model where G-protein coupled receptors (GPCRs) isomerize between inactive and active states [54, 55]. New concepts in GPCR signaling, termed ligand biased signaling, considers that various ligands may result in a variety of conformational changes to GPCRs that result in activation of different G-protein signaling pathways. For example the adenine A1 receptor can adopt agonist-specific conformations arising from small changes in ligand structure which result in differential activation of G<sub>i</sub>, G<sub>s</sub> and G<sub>q</sub> [56]. The CaSR is known to couple to G<sub>q</sub>, G<sub>i</sub> and G<sub>s</sub> signaling proteins [19, 20] all of which are in the proximal tubule [21, 22]. Though many GPCRs, including the CaSR, are known to activate more than one G-protein at a time, ligand biased signaling involves strong bias toward one pathway at the expense of others that may be activated under alternative conditions.

Taken together, the results of spermine, thapsigargin and PMA experiments (Figs. 2, 3, 4) show that apical dicarboxylate transport inhibition in normal [Ca<sup>2+</sup>]<sub>o</sub> occurs via the CaSR → G<sub>q</sub> → PKC pathway depicted in Fig. 1. Results with PTX and 8Br on transport (Figs. 5, 7, 8), and the measurements of cAMP (Fig. 9), indicate that changes in intracellular cAMP via G<sub>s</sub> and G<sub>i</sub> are not likely involved in regulation of dicarboxylate transport by the CaSR.

In summary, in OK cells CaSR activation (even by normal [Ca<sup>2+</sup>]<sub>o</sub>) inhibits dicarboxylate transport via G<sub>q</sub> signaling. Our previous studies have consistently demonstrated calcium-sensitive dicarboxylate transport process in OK proximal tubule cells. Here we offer evidence that CaSR → G<sub>q</sub> → PKC signal transduction pathway regulates apical dicarboxylate transport. This pathway provides a mechanism whereby the proximal tubule apical CaSR prevents calcium precipitation by reducing apical dicarboxylate transport.

**Acknowledgements** This work was supported by NIH/NIDDK RO1 DK095879 to Dr. Hering-Smith.

**Funding** This study was funded by NIH/NIDDK RO1 DK095879 to Dr. Hering-Smith. Dr. Hering-Smith is also supported by the NIH Louisiana Clinical and Translational Sciences Center (LA CaTS) grant.

## Compliance with ethical standards

**Conflict of interest** Dr. Ryan Walker declares that he has no conflict of interest. Dr. Shijia Zhang declares that he has no conflict of interest. Ms. Joycelynn Coleman-Barnett declares that she has no conflict of interest. Dr. L. Lee Hamm declares that he has no conflict of interest. Dr. Kathleen Hering-Smith declares that she has no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

## References

1. Egbuna O, Quinn S, Kantham L, Butters R, Pang J, Pollak M, Goltzman D, Brown E (2009) The full-length calcium-sensing receptor dampens the calcemic response to 1α,25(OH)<sub>2</sub> vitamin D<sub>3</sub> in vivo independently of parathyroid hormone. *Am J Physiol Renal Physiol* 297(3):F720–F728
2. Quamme GA (1982) Effect of hypercalcemia on renal tubular handling of calcium and magnesium. *Can J Physiol Pharmacol* 60(10):1275–1280
3. Riccardi D, Brown EM (2010) Physiology and pathophysiology of the calcium-sensing receptor in the kidney. *Am J Physiol Renal Physiol* 298(3):F485–F499
4. Wang WH, Lu M, Hebert SC (1996) Cytochrome P-450 metabolites mediate extracellular Ca<sup>2+</sup>-induced inhibition of apical K<sup>+</sup> channels in the TAL. *Am J Physiol* 271(1 Pt 1):C103–C111
5. Topala CN, Schoeber JP, Searchfield LE, Riccardi D, Hoenderop JG, Bindels RJ (2009) Activation of the Ca<sup>2+</sup>-sensing receptor stimulates the activity of the epithelial Ca<sup>2+</sup> channel TRPV5. *Cell Calcium* 45(4):331–339
6. Renkema KY, Velic A, Dijkman HB, Verkaart S, van der Kemp AW, Nowik M, Timmermans K, Doucet A, Wagner CA, Bindels RJ, Hoenderop JG (2009) The calcium-sensing receptor promotes urinary acidification to prevent nephrolithiasis. *J Am Soc Nephrol* 20(8):1705–1713
7. Riccardi D, Hall AE, Chattopadhyay N, Xu JZ, Brown EM, Hebert SC (1998) Localization of the extracellular Ca<sup>2+</sup>/polyvalent cation-sensing protein in rat kidney. *Am J Physiol* 274(3 Pt 2):F611–F622
8. Conigrave AD, Ward DT (2013) Calcium-sensing receptor (CaSR): pharmacological properties and signaling pathways. *Best Pract Res Clin Endocrinol Metab* 27(3):315–331
9. Remy C, Kirchhoff P, Hafner P, Busque SM, Müller MK, Geibel JP, Wagner CA (2007) Stimulatory pathways of the calcium-sensing receptor on acid secretion in freshly isolated human gastric glands. *Cell Physiol Biochem* 19(1–4):33–42
10. Capasso G, Geibel PJ, Damiano S, Jaeger P, Richards WG, Geibel JP (2013) The calcium sensing receptor modulates fluid reabsorption and acid secretion in the proximal tubule. *Kidney Int* 84(2):277–284
11. Pajor AM (1999) Sodium-coupled transporters for Krebs cycle intermediates. *Annu Rev Physiol* 61:663–682



12. Bai XY, Chen X, Sun AQ, Feng Z, Hou K, Fu B (2007) Membrane topology structure of human high-affinity, sodium-dependent dicarboxylate transporter. *FASEB J* 21(10):2409–2417
13. Pajor AM (1999) Citrate transport by the kidney and intestine. *Semin Nephrol* 19(2):195–200
14. Brennan TS, Klahr S, Hamm LL (1986) Citrate transport in rabbit nephron. *Am J Physiol* 251(4 Pt 2):F683–F689
15. Hering-Smith KS, Gambala CT, Hamm LL (2000) Citrate and succinate transport in proximal tubule cells. *Am J Physiol Renal Physiol* 278(3):F492–F498
16. Hering-Smith KS, Schiro FR, Pajor AM, Hamm LL (2011) Calcium sensitivity of dicarboxylate transport in cultured proximal tubule cells. *Am J Physiol Renal Physiol* 300(2):F425–F432
17. Hering-Smith KS, Mao W, Schiro FR, Coleman-Barnett J, Pajor AM, Hamm LL (2014) Localization of the calcium-regulated citrate transport process in proximal tubule cells. *Urolithiasis* 42(3):209–219
18. Law D, Hering-Smith KS, Hamm LL (1992) Citrate transport in proximal cell line. *Am J Physiol* 263(1 Pt 1):C220–C225
19. Hofer AM, Brown EM (2003) Extracellular calcium sensing and signalling. *Nat Rev Mol Cell Biol* 4(7):530–538
20. Hernández-Bedolla MA, González-Domínguez E, Zavala-Barraera C, Gutiérrez-López TY, Hidalgo-Moyle JJ, Vázquez-Prado J, Sánchez-Torres C, Reyes-Cruz G (2016) Calcium-sensing-receptor (CaSR) controls IL-6 secretion in metastatic breast cancer MDA-MB-231 cells by a dual mechanism revealed by agonist and inverse-agonist modulators. *Mol Cell Endocrinol* 436:159–168
21. Zhu Y, He Q, Aydin C, Rubera I, Tauc M, Chen M, Weinstein LS, Marshansky V, Jüppner H, Bastepe M (2016) Ablation of the stimulatory G protein  $\alpha$ -subunit in renal proximal tubules leads to parathyroid hormone-resistance with increased renal Cyp24a1 mRNA abundance and reduced serum 1,25-dihydroxyvitamin D. *Endocrinology* 157(2):497–507
22. Rangel LB, Lopes AG, Lara LS, Carvalho TL, Silva IV, Oliveira MM, Einicker-Lamas M, Vieyra A, Nogaroli L, Caruso-Neves C (2005) PI-PLC $\beta$  is involved in the modulation of the proximal tubule Na<sup>+</sup>-ATPase by angiotensin II. *Regul Pept* 127(1–3):177–182
23. Wu DQ, Lee CH, Rhee SG, Simon MI (1992) Activation of phospholipase C by the alpha subunits of the Gq and G11 proteins in transfected Cos-7 cells. *J Biol Chem* 267(3):1811–1817
24. Nasman J, Kukkonen JP, Ammoun S, Akerman KE (2001) Role of G-protein availability in differential signaling by alpha 2-adrenoceptors. *Biochem Pharmacol* 62(7):913–922
25. Brennan SC, Thiem U, Roth S, Aggarwal A, Fetahu IS, Tennakoon S, Gomes AR, Brandi ML, Bruggeman F, Mentaverrì R, Riccardi D, Kallay E (2013) Calcium sensing receptor signalling in physiology and cancer. *Biochim Biophys Acta* 1833(7):1732–1744
26. Petrel C, Kessler A, Dauban P, Dodd RH, Rognan D, Ruat M (2004) Positive and negative allosteric modulators of the Ca<sup>2+</sup>-sensing receptor interact within overlapping but not identical binding sites in the transmembrane domain. *J Biol Chem* 279(18):18990–18997
27. Harriman JF, Liu XL, Aleo MD, Machaca K, Schnellmann RG (2002) Endoplasmic reticulum Ca(2<sup>+</sup>) signaling and calpains mediate renal cell death. *Cell Death Differ* 9(7):734–741
28. Beckman ML, Bernstein EM, Quick MW (1999) Multiple G protein-coupled receptors initiate protein kinase C redistribution of GABA transporters in hippocampal neurons. *J Neurosci* 19(11):RC9
29. Forster IC, Traebert M, Jankowski M, Stange G, Biber J, Murer H (1999) Protein kinase C activators induce membrane retrieval of type II Na<sup>+</sup>-phosphate cotransporters expressed in *Xenopus oocytes*. *J Physiol* 517(Pt 2):327–340
30. Emery AC, Eiden MV, Eiden LE (2013) A new site and mechanism of action for the widely used adenylate cyclase inhibitor SQ22,536. *Mol Pharmacol* 83:95–105
31. Yamaguchi T et al (2003) Cyclic AMP activates B-Raf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys. *Kidney Int* 63:1983–1994
32. Siebler T et al (1996) Pertussis toxin sensitive G-proteins are not involved in the mitogenic signaling pathway of insulin-like growth factor-I in normal rat kidney epithelial (NRKE) cells. *Regul Pept* 62:65–71
33. Wang D, McGiff JC, Ferreri NR (2000) Regulation of cyclooxygenase isoforms in the renal thick ascending limb: effects of extracellular calcium. *J Physiol Pharmacol* 51(4 Pt 1):587–595
34. Kifor O, MacLeod RJ, Diaz R, Bai M, Yamaguchi T, Yao T, Kifor I, Brown EM (2001) Regulation of MAP kinase by calcium-sensing receptor in bovine parathyroid and CaR-transfected HEK293 cells. *Am J Physiol Renal Physiol* 280(2):F291–F302
35. Kempson SA, Edwards JM, Sturek M (2006) Inhibition of the renal betaine transporter by calcium ions. *Am J Physiol Renal Physiol* 291(2):F305–F313
36. Hagos Y, Burckhardt BC, Larsen A, Mathys C, Gronow T, Bahn A, Wolff NA, Burckhardt G, Steffgen J (2004) Regulation of sodium-dicarboxylate cotransporter-3 from winter flounder kidney by protein kinase C. *Am J Physiol Renal Physiol* 286(1):F86–F93
37. Wolff NA, Thies K, Kuhnke N, Reid G, Friedrich B, Lang F, Burckhardt G (2003) Protein kinase C activation downregulates human organic anion transporter 1-mediated transport through carrier internalization. *J Am Soc Nephrol* 14(8):1959–1968
38. Pajor AM, Sun N (1999) Protein kinase C-mediated regulation of the renal Na<sup>+</sup>/dicarboxylate cotransporter, NaDC-1. *Biochim Biophys Acta* 1420(1–2):223–230
39. Collins TP, Terrar DA (2012) Ca(2<sup>+</sup>)-stimulated adenylyl cyclases regulate the L-type Ca(2<sup>+</sup>) current in guinea-pig atrial myocytes. *J Physiol* 590(8):1881–1893
40. Li X, Guo Q, Gao J, Yang J, Zhang W, Liang Y, Wu D, Liu Y, Weng J, Li Q, Zhang Y (2013) The adenylyl cyclase inhibitor MDL-12,330A potentiates insulin secretion via blockade of voltage-dependent K(+) channels in pancreatic beta cells. *PLoS One* 8(10):e77934
41. Dal Molin F, Zornetta I, Puhar A, Tonello F, Zaccolo M, Montecucco C (2008) cAMP imaging of cells treated with pertussis toxin, cholera toxin, and anthrax edema toxin. *Biochem Biophys Res Commun* 376(2):429–433
42. Gadea A, López E, López-Colomé AM (1999) The adenylate cyclase inhibitor MDL-12330A has a non-specific effect on glycine transport in Müller cells from the retina. *Brain Res* 838(1–2):200–204
43. Lippe C, Ardizzone C (1991) Actions of vasopressin and isoprenaline on the ionic transport across the isolated frog skin in the presence and the absence of adenylyl cyclase inhibitors MDL12330A and SQ22536. *Comp Biochem Physiol C* 99(1–2):209–211
44. Rampe D, Triggle DJ, Brown AM (1987) Electrophysiologic and biochemical studies on the putative Ca<sup>++</sup> channel blocker MDL 12,330A in an endocrine cell. *J Pharmacol Exp Ther* 243(1):402–407
45. Brennan S, Hering-Smith K, Hamm LL (1988) Effect of pH on citrate reabsorption in the proximal convoluted tubule. *Am J Physiol* 255(2 Pt 2):F301–306
46. Hamm LL, Hering-Smith KS (2002) Pathophysiology of hypocitraturic nephrolithiasis. *Endocrinol Metab Clin North Am* 31(4):885–893 viii
47. Peti-Peterdi J (2013) Mitochondrial TCA cycle intermediates regulate body fluid and acid–base balance. *J Clin Invest* 123(7):2788–2790
48. Magno AL, Ward BK, Ratajczak T (2011) The calcium-sensing receptor: a molecular perspective. *Endocr Rev* 32(1):3–30

49. Chang W, Shoback D (2004) Extracellular Ca<sup>2+</sup>-sensing receptors—an overview. *Cell Calcium* 35(3):183–196
50. Kenakin T (2011) Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther* 336(2):296–302
51. Thomsen AR, Hvidtfeldt M, Bräuner-Osborne H (2012) Biased agonism of the calcium-sensing receptor. *Cell Calcium* 51(2):107–116
52. Thomsen AR, Worm J, Jacobsen SE, Stahlhut M, Latta M, Bräuner-Osborne H (2012) Strontium is a biased agonist of the calcium-sensing receptor in rat medullary thyroid carcinoma 6–23 cells. *J Pharmacol Exp Ther* 343(3):638–649
53. Davey AE, Leach K, Valant C, Conigrave AD, Sexton PM, Christopoulos A (2012) Positive and negative allosteric modulators promote biased signaling at the calcium-sensing receptor. *Endocrinology* 153(3):1232–1241
54. Samama P, Cotecchia S, Costa T, Lefkowitz RJ (1993) A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* 268(7):4625–4636
55. Gether U, Lin S, Kobilka BK (1995) Fluorescent labeling of purified beta 2 adrenergic receptor. Evidence for ligand-specific conformational changes. *J Biol Chem* 270(47):28268–28275
56. Cordeaux Y, Ijzerman AP, Hill SJ (2004) Coupling of the human A1 adenosine receptor to different heterotrimeric G proteins: evidence for agonist-specific G protein activation. *Br J Pharmacol* 143(6):705–714