

## Discussion Letter

 $\text{Na}^+/\text{H}^+$  exchange and  $\text{Ca}^{2+}$  influxWinfried Siffert and Jan Willem N. Akkerman<sup>+</sup>*Max-Planck-Institut für Biophysik, Kennedyallee 70, D-6000 Frankfurt am Main 70, FRG and <sup>+</sup>Department of Haematology, University Hospital, 3511 GV Utrecht, The Netherlands*

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Cell stimulation raises the cytosolic free  $\text{Ca}^{2+}$  concentration,  $[\text{Ca}^{2+}]_i$ , and induces activation of  $\text{Na}^+/\text{H}^+$  exchange which raises the cytosolic pH,  $\text{pH}_i$ . Recent studies have addressed the question whether  $\text{Na}^+/\text{H}^+$  exchange plays a role in  $\text{Ca}^{2+}$  influx and, specifically, whether a rise in  $\text{pH}_i$  alone suffices to open  $\text{Ca}^{2+}$  channels in the plasma membrane. Artificial cytosolic alkalinization can induce  $\text{Ca}^{2+}$  uptake across the plasma membrane of endothelial cells, lymphocytes and smooth muscle cells. Furthermore, inhibition of  $\text{Na}^+/\text{H}^+$  exchange reduces agonist-induced  $\text{Ca}^{2+}$  influx in endothelial cells and platelets which supports the concept that  $\text{pH}_i$  may regulate the opening of  $\text{Ca}^{2+}$  channels in the plasma membrane. Although these findings argue in favour of a role of  $\text{Na}^+/\text{H}^+$  exchange in  $\text{Ca}^{2+}$  influx, the onset of  $\text{pH}_i$  and  $\text{Ca}^{2+}$  rises, measured with fluorescent indicators, suggests that the increase in  $[\text{Ca}^{2+}]_i$  distinctly precedes the increase in  $\text{pH}_i$ . This challenges the concept that alkalinization per se is a sufficient signal for the opening of  $\text{Ca}^{2+}$  channels in the plasma membrane.

Platelet; Endothelial cell; Lymphocyte; Smooth muscle cell;  $\text{Na}^+/\text{H}^+$  exchange

## 1. INTRODUCTION

All eukaryotic cells possess an ion transport system in their plasma membrane which, driven by the inwardly directed  $\text{Na}^+$  gradient, extrudes  $\text{H}^+$  from the cytosol to the extracellular space. This ion transport system is commonly referred to as the ' $\text{Na}^+/\text{H}^+$  exchanger' or ' $\text{Na}^+/\text{H}^+$  antiport'.

In resting cells the  $\text{Na}^+/\text{H}^+$  exchanger is activated by an increase in the intracellular  $\text{H}^+$  concentration and replaces intracellular  $\text{H}^+$ -ions for external  $\text{Na}^+$ -ions until the original cytoplasmic pH,  $\text{pH}_i$ , of approximately 7.1 (at  $\text{pH}_o$  7.4) is re-established. This recovery of  $\text{pH}_i$  from cytosolic acidification can be prevented by either removal of  $\text{Na}^+$  ions from the extracellular space or by incubation of cells with amiloride and its analogs. Hence, in unstimulated cells the role of the antiport is to keep  $\text{pH}_i$  constant and to counteract cytosolic acidification [1-4]. In contrast, when cells are activated by specific stimuli such as growth factors or hormones,  $\text{pH}_i$  increases to ranges close to the extracellular value

of 7.4. This increase in  $\text{pH}_i$  is also abolished in  $\text{Na}^+$ -free medium and by amiloride analogs which indicates the involvement of  $\text{Na}^+/\text{H}^+$  exchange in this process. It is generally believed that cell stimulation induces certain modifications in the exchanger that enhance its affinity towards internal  $\text{H}^+$  [1-5]. Blocking  $\text{Na}^+/\text{H}^+$  exchange does not only prevent the agonist-induced increase in  $\text{pH}_i$ , but also reduces various cell functions (for review see [6-8]). It should be explicitly noted, however, that most of the above-cited studies have been conducted in nominally bicarbonate-free media, and that some cell lines have been reported not to respond with cytosolic alkalinization to stimulation by agonists in the presence of bicarbonate (for review see [3]).

The present review focuses on the potential interrelationship between rises in  $\text{pH}_i$  and  $[\text{Ca}^{2+}]_i$  in stimulated cells with special emphasis on the question whether or not a rise in  $\text{pH}_i$  alone is sufficient to trigger an influx of  $\text{Ca}^{2+}$  ions.

2. IS A RISE IN  $\text{pH}_i$  ALONE SUFFICIENT TO INCREASE  $[\text{Ca}^{2+}]_i$ ?

One of the earliest events in stimulated cells is a rapid rise in  $[\text{Ca}^{2+}]_i$ . Part of this  $\text{Ca}^{2+}$  stems from intracellular storage sites and is released through the action of inositol 1,4,5-trisphosphate ( $\text{Ins } 1,4,5\text{P}_3$ ) [9-11]. However, the  $\text{Ins } 1,4,5\text{P}_3$ -induced  $\text{Ca}^{2+}$  mobilization is

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*Abbreviations:*  $[\text{Ca}^{2+}]_i$ , cytosolic free  $\text{Ca}^{2+}$  concentration;  $\text{Ca}^{2+}_o$ , extracellular  $\text{Ca}^{2+}$ ; EIPA, ethylisopropylamiloride;  $[\text{H}^+]_i$ , cytosolic  $\text{H}^+$  concentration;  $[\text{Na}^+]_i$ , cytosolic  $\text{Na}^+$  concentration;  $\text{Na}^+_o$ , extracellular  $\text{Na}^+$ ;  $\text{pH}_i$ , cytosolic pH;  $\text{pH}_o$ , extracellular pH;  $\text{Ins } 1,4,5\text{P}_3$ , inositol 1,4,5 trisphosphate

Table 1

Effect of artificial cytosolic alkalinization and agonists on  $[Ca^{2+}]_i$  in the presence of extracellular  $Ca^{2+}$ , i.e.  $Ca^{2+}$  influx plus mobilization from intracellular stores

Cell type	NH <sub>4</sub> Cl		Monensin		Agonist		Ref.
	$\Delta pH_i$	$\Delta [Ca^{2+}]_i$ (nM)	$\Delta pH_i$	$\Delta [Ca^{2+}]_i$ (nM)	$\Delta pH_i$	$\Delta [Ca^{2+}]_i$ (nM)	
Endothelial cells	0.34	1410	0.16	1164	0.36	700	15
Lymphocytes	0.32	119	0.26	178	n.d.	n.d.	13
Platelets	0.27	400	0.31	420	0.36	1000	16
Platelets	n.d.	n.d.	0.60	0	0.25	270	18
Platelets	0.27	0	n.d.	n.d.	(0)	1000	17
Smooth muscle cells	0.41	46	n.d.	n.d.	n.d.	n.d.	19

Shown are the rises in  $pH_i$  and  $[Ca^{2+}]_i$  evoked by natural agonists (where available) in comparison with changes in  $[Ca^{2+}]_i$  after artificially elevating  $pH_i$  by either NH<sub>4</sub>Cl or monensin. Endothelial cells and platelets were activated by thrombin, smooth muscle cells by arginine-vasopressin. In lymphocytes, no data for agonist-induced changes in  $pH_i$  and  $[Ca^{2+}]_i$  are available in the same study. Since all  $Ca^{2+}$  measurements were conducted in the presence of extracellular  $Ca^{2+}$ , changes in  $[Ca^{2+}]_i$  represent the influx of  $Ca^{2+}$  ions from the extracellular space to the cytosol plus mobilization from intracellular stores. All values represent increases of  $pH_i$  (units) and  $[Ca^{2+}]_i$  (nM) above untreated controls

only transient and, in the absence of extracellular  $Ca^{2+}$ ,  $Ca^{2+}_0$ ,  $[Ca^{2+}]_i$  rapidly returns to resting levels. Under physiological conditions, i.e. in the presence of  $Ca^{2+}_0$ , the elevation of  $[Ca^{2+}]_i$  lasts much longer as a result of an influx of  $Ca^{2+}$  across the plasma membrane. The exact mechanism by which agonists promote  $Ca^{2+}$  influx remains to be defined. Since most non-excitabile cells lack voltage-dependent  $Ca^{2+}$  channels, one may presume that the entry of  $Ca^{2+}$  is mediated via receptor-operated  $Ca^{2+}$  channels [12]. An important question is whether cytoplasmic alkalinization alone is a sufficient signal for  $Ca^{2+}$  influx. This question has been addressed in different cell types, e.g. lymphocytes, endothelial cells, smooth muscle cells and platelets. In these studies  $pH_i$  was artificially elevated by either addition of NH<sub>4</sub>Cl or the use of the  $Na^+/H^+$  ionophore monensin. The effect of these manipulations on  $[Ca^{2+}]_i$  was assessed from quin2 or fura2 fluorescence, and the translocation of  $^{45}Ca^{2+}$ . Since the  $Ca^{2+}$ -binding properties of quin2 and fura2 are not affected by variations of pH between 7.0 and 7.5 [13,14], the changes in fluorescence seen after artificial cytosolic alkalinization are accurate reflections of  $[Ca^{2+}]_i$ . Table 1 summarizes the effects of artificially elevating  $pH_i$  on  $[Ca^{2+}]_i$  in different cell types, and compares these changes with those induced by natural agonists in the same studies (as far as data are available). As these experiments have been conducted in the presence of extracellular  $Ca^{2+}$ , both  $Ca^{2+}$  mobilization and influx add to the net rise in  $[Ca^{2+}]_i$ . Ghigo et al. observed that treatment of endothelial cells with either NH<sub>4</sub>Cl or monensin evoked a strong influx of  $Ca^{2+}$  which even exceeded that induced by thrombin [15]. Similarly, these manipulations induced a significant influx of  $Ca^{2+}$  in quin2-loaded lymphocytes [13] which was completely abolished after chelating  $Ca^{2+}_0$  by addition of EGTA. The observation that  $Ca^{2+}$  influx was not only induced by monensin (which apart from raising  $pH_i$  also elevates  $[Na^+]_i$ ), but also by

NH<sub>4</sub>Cl treatment (which raises  $pH_i$  without altering  $[Na^+]_i$ ) points at the rise in  $pH_i$  – rather than the change in  $[Na^+]_i$  – as the cause of this response. The findings in platelets are controversial. Ghigo et al. [16] reported that artificially elevating  $pH_i$  by both monensin and NH<sub>4</sub>Cl induced an influx of  $Ca^{2+}$  amounting to almost 50% of that evoked by thrombin. This observation was confirmed by measuring monensin-induced uptake of  $^{45}Ca^{2+}$  [16]. In contrast, both Simpson and Rink [17] as well as our group [18] failed to detect an influx of  $Ca^{2+}$  in response to cytosolic alkalinization along in quin2- or fura2-loaded platelets. Raising  $pH_i$  by NH<sub>4</sub>Cl in smooth muscle cells induced some minor albeit significant  $Ca^{2+}$  influx, and the change in  $[Ca^{2+}]_i$  was twice that observed in the absence of  $Ca^{2+}_0$  [19]. These observations argue in favour of a role of alkalinization in  $Ca^{2+}$  entry in these cells. Unfortunately, the precise mechanisms underlying this process remain obscure. In theory, two different mechanisms may be involved. First, a rise in  $pH_i$  might directly open receptor-operated  $Ca^{2+}$  channels, e.g. by altering the protonation of the permeation site, thereby allowing  $Ca^{2+}$  influx. No data in support of this hypothesis are yet available. Second, alkalinization could induce the intracellular formation of substances which, upon release, combine with specific receptors on the cell surface subsequently promoting agonist-induced  $Ca^{2+}$  influx. Possible candidates are certain prostaglandins, products of the phospholipase A<sub>2</sub> pathway, or platelet-activating factor, which are formed via pH-dependent processes [15,20–22].

### 3. IS INTACT $Na^+/H^+$ EXCHANGE REQUIRED FOR STIMULUS-INDUCED $Ca^{2+}$ INFLUX?

One means to investigate the contribution of  $Na^+/H^+$  exchange to stimulus-induced influx of  $Ca^{2+}$  is to measure changes in fluorescence of quin2- or

Table 2

Effect of inhibition of  $\text{Na}^+/\text{H}^+$  exchange in different cell types on stimulus-induced  $[\text{Ca}^{2+}]_i$  rises in the presence of extracellular  $\text{Ca}^{2+}$ , i.e.  $\text{Ca}^{2+}$  influx plus mobilization from intracellular stores

Cell type	EIPA $\Delta[\text{Ca}^{2+}]_i$ (% of control)	$\text{Na}_0^+$ removal $\Delta[\text{Ca}^{2+}]_i$ (% of control)	Stimulus	Ref.
Endothelial cells	0	0	Thr	15
Platelets	n.d.	37	Thr (0.1 U/ml)	16
Platelets	n.d.	36	AA	16
Platelets	40	15	Thr (0.2 U/ml)	25
Platelets	0	n.d.	Thr (0.5 U/ml)	27
Platelets	74	16	Thr (0.1–0.5 U/ml)	26
Platelets	n.d.	100	Thr (0.5 U/ml)	23
Smooth muscle cells	n.d.	100	AII	28

Shown is the effect of inhibiting  $\text{Na}^+/\text{H}^+$  exchange by either ethylisopropylamiloride (EIPA) or  $\text{Na}^+$  substitution on stimulus-induced  $\text{Ca}^{2+}$  influx. Changes in  $[\text{Ca}^{2+}]_i$  were deduced from changes in fluorescence of fura2- or quin2-loaded cells. The values represent the rises in  $[\text{Ca}^{2+}]_i$  (% of control) observed after blockade of  $\text{Na}^+/\text{H}^+$  exchange. Thr, thrombin; AA, arachidonic acid; AII, angiotensin II. The values in parentheses indicate the agonist concentrations used for cell stimulation

fura2-loaded cells after blocking  $\text{Na}^+/\text{H}^+$  exchange by either iso-osmotic substitution of  $\text{Na}_0^+$  or by direct inhibition of  $\text{Na}^+/\text{H}^+$  exchange with amiloride analogs. One potential drawback of this method is that this treatment does not only prevent cytosolic alkalinization but also causes dramatic cytosolic acidification in cells stimulated by agonists. Hence, it may be difficult to decide whether an impairment of  $\text{Ca}^{2+}$  influx by EIPA or removal of  $\text{Na}_0^+$  is caused by a fall in  $\text{pH}_i$  to below the resting value or actually by prevention of cytosolic alkalinization. Most of these studies have been performed in platelets and only a few reports on other cell types have been published. An overview of these experiments is given in table 2. Studies in endothelial cells demonstrated that inhibition of  $\text{Na}^+/\text{H}^+$  exchange inhibited thrombin-stimulated  $\text{Ca}^{2+}$  influx as assessed from quin2 fluorescence and the influx of  $^{45}\text{Ca}^{2+}$  [15]. This observation agrees well with the proposed role of alkalinization as an inducer of  $\text{Ca}^{2+}$  influx (cf. table 1). Both  $\text{Na}_0^+$  removal as well as EIPA also lowered  $\text{Ca}^{2+}$  influx in platelets stimulated by thrombin or arachidonic acid [16]. Except reports by Sage and Rink [23] and Sanchez et al. [24] most studies demonstrated that intact  $\text{Na}^+/\text{H}^+$  exchange contributes to agonist-induced  $\text{Ca}^{2+}$  uptake in human platelets (e.g. [25–27]). Hence, in this respect platelets behave similarly to endothelial cells. In smooth muscle cells, on the other hand,  $\text{Na}_0^+$  removal apparently had no effect on angiotensin II-induced  $\text{Ca}^{2+}$  influx [25], although the finding that artificial cytosolic alkalinization induces  $\text{Ca}^{2+}$  uptake would predict a role of  $\text{Na}^+/\text{H}^+$  exchange.

#### 4. CONCLUSIONS

There is increasing evidence that intact  $\text{Na}^+/\text{H}^+$  exchange is required for the mechanisms that evoke  $\text{Ca}^{2+}$  influx in stimulated cells. Although an artificially produced rise in  $\text{pH}_i$  may suffice to trigger  $\text{Ca}^{2+}$  influx in

endothelial cells, lymphocytes, and smooth muscle cells it is doubtful whether a similar mechanism is operative in agonist-stimulated cells. Especially the observation that maximum  $\text{Ca}^{2+}$  influx precedes the rise in  $\text{pH}_i$  makes it unlikely that cytosolic alkalinization per se works as a 'second messenger' at receptor-operated  $\text{Ca}^{2+}$  channels. On the other hand, the reports showing a strong reduction of  $\text{Ca}^{2+}$  influx after blockade of  $\text{Na}^+/\text{H}^+$  exchange strongly suggest the involvement of a pH-sensitive step in the opening of  $\text{Ca}^{2+}$  channels. It remains to be clarified whether the role of  $\text{Na}^+/\text{H}^+$  exchange is restricted to merely prevent cytosolic acidification in stimulated cells, or whether the observed increases in cytosolic pH also contribute to the mechanisms allowing for  $\text{Ca}^{2+}$  influx across the plasma membrane.

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