BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **242,** 123–128 (1998)
ARTICLE NO. RC977927

Extracellular Acidification Modifies Ca^{2+} Fluxes in Rat Brain Synaptosomes

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Received November 14, 1997

We examined the influence of external acidification on Ca^{2+} fluxes ($^{45}Ca^{2+}$ influx and $^{45}Ca^{2+}$ efflux) in rat brain synaptosomes. A change on external pH (pH_e) from 7.5 to 6.5 linearly decreased the $^{45}Ca^{2+}$ uptake (5nmoles/mg protein/pHunit) and increased the $^{45}Ca^{2+}$ efflux (1.5 fold/pH unit). These changes were both Na^+ dependent and amiloride sensitive suggesting that the Na^+/Ca^{2+} exchanger could be involved. The addition of the Ca^{2+} channel blockers (diltiazem, verapamil, nifedipine) did not prevent the decrease of the $^{45}Ca^{2+}$ uptake evoked by acid pH_e and so the involvement of the voltage-sensitive Ca^{2+} channels could be discarded. In order to determine whether the Na^+/Ca^{2+} exchanger was directly activated by H^+ or was indirectly activated by an internal mobilization of Ca^{2+} from intrasynaptosomal stores we examined the effect of pH_e variation on phophoinositide hydrolisis. An increase on phosphoinositide hydrolisis was observed at acid pH_e values (7 and 6.5). The hydrolisis was amiloride insensitive. On the other hand 1mM neomycin did inhibit the effect of acidic pH_e on Ca^{2+} fluxes. Taken together, the results of our study provide evidence that external acidification stimulates phospholipase C leading to an increase in phosphoinositide hydrolisis and Ca^{2+} mobilization. The increase in intracellular Ca^{2+} would stimulate the Na^+/Ca^{2+} exchanger, increasing Ca^{2+} efflux and reducing the global Ca^{2+} influx.

Key Words: **synaptosomes; external pH; acidification; Ca^{2+} fluxes; Na^+/Ca^{2+} exchanger; voltage sensitive calcium channels; phosphoinositide hydrolisis.**

External acidification or alkalinitation could be produced under physiological and pathological conditions in the central nervous system (1-3). It has been described that this change in external pH (pH_e) affects Ca^{2+} metabolism in a variety of cells (4-6). However, relatively little information is available on how a change in pH_e could influence Ca^{2+} metabolism. In this respect, several evidencies have indicated that pH_e could alter Ca^{2+} homeostasis by modulating the flux of Ca^{2+} across plasma membrane and/or the Ca^{2+} release by intracellular organelles (4,6,7). However the precise mechanisms involved are still poorly known. On the other hand, it has been shown that protons could compite with Ca^{2+} for the binding to proteins or other macromolecules (8,9), producing an increase in the intracellular Ca^{2+} concentration.

In several pathological situations like ischemia or anoxia, neuronal death is believed to occur by an increase in the release of glutamate by the presynaptic terminal (10,11). The increase in the extracellular concentration of glutamate will overstimulate certain types of glutamate receptors, starting a cellular cascade that will eventualy produce neuronal death (12,13). Several evidencies have shown that mild acidosis of the extracellular medium has a neuroprotective effect against glutamate-mediated neurotoxicity (14,15). Since neurotransmitter release is dependent on presynaptic Ca^{2+} levels, it is possible to think that the protective effect observed during mild acidosis could be explained by an effect on the Ca^{2+} homeostasis in the presynaptic terminal. In this respect, it has been described that external acidification inhibits presynaptic Ca^{2+} entry evoked by depolarization with KCl (16) and glutamate-mediated increase in intracellular Ca^{2+} (4,7).

In the presynaptic terminal Ca^{2+} homeostasis is mainly controlled by three mechanisms: 1) influx through voltage-sensitive Ca^{2+} channels (VSCC; 17); 2) efflux by the activity of the Na^+/Ca^{2+} exchanger and the Ca^{2+}-pump located in the plasma membrane (18,19); 3) mobilization from intracellular stores (20,21).

The aim of the present study was to elucidate the mechanisms involved in the control of Ca^{2+} homeostasis in the presynaptic terminal by pH_e. We have explored how changes in pH_e affect Ca^{2+} influx and Ca^{2+} efflux in rat brain synaptosomes and the eventual involvement of VSCC, the Ca^{2+}-pump and the Na^+/Ca^{2+} exchanger.

Abbreviations: pH_e, external pH; VSSC, voltage sensitive calcium channels.

0006-291X/98 $25.00

MATERIAL AND METHODS

Chemicals. Amiloride was obtained from the Sigma Chemical Co. $^{45}Ca^{2+}$ and [^{3}H]-inositol were obtained from New England Nuclear. Neomycin was kindly supplied by Laboratorios J. Uriach & Cia. (Spain). All other reagents were of analytical grade.

Isolation of synaptosomes. Synaptosomes were prepared from rat brains as described by Dodd *et al.*, (22). The brains were homogenized at 10% (wt/vol) in 0.32M sucrose buffered with 20mM Tris-HCl, pH 7.5, using a teflon pestle in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 1000 ×g for 10 min. 8 ml supernatant were layered onto 4ml of 1.2 M sucrose and centrifuged at 112000 ×g for 15 min. The interface between 0.32-1.2 M sucrose was collected and layered onto 4ml of 0.8M sucrose and centrifuged at 112000 ×g for 15 min. The synaptosomal pellet was suspended in 0.32 M sucrose, 20 mM Tris-HCl, pH 7.5, at a concentration of 10-20 mg protein/ml for $^{45}Ca^{2+}$ influx studies. For $^{45}Ca^{2+}$ efflux studies synaptosomal pellet was resuspended in Ringer-Tris, or test medium, at pH 7.5 adjusted with HCl at a concentration of 0.5-1.0 mg protein/ml. Ringer-Tris medium consisted of 130 mM NaCl, 1.4 mM NaH_2PO_4, 5.0 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 20 mM Tris-HCl, 10 mM glucose. When the effects of the Na^+-driven mechanisms were determined, NaCl was replaced by 130mM choline chloride. Protein content was determined by the Lowry method (23) using bovine serum albumin as a standard. Lactate dehydrogenase activity was determined to check the integrity of the synaptosomal preparation (24). The enzyme activity did not change when pH_e was reduced from 7.5 to 6.5.

$^{45}Ca^{2+}$ influx. $^{45}Ca^{2+}$ influx was carried out by the method previously described by Rodriguez et al., (25). Synaptosomal suspension (25 μl) was preincubated for 20 min at 37°C with 200 μl at different pH values from 7.5 to 6.5. $^{45}Ca^{2+}$ influx was started by the addition of 0.3 μCi $^{45}Ca^{2+}$ (1.0 μCi/μmol Ca^{2+}) and stopped one minute after by a 10-fold dilution of the samples with ice-cold stopped buffer (Ringer-Tris buffer supplemented with 5mM La^{3+}, pH 7.5), followed by rapid filtration through GF/C Whatman glass filters prewashed with 10ml of stopped buffer on a Millipore vacuum filtration manifold. After three separate washes, filters were placed in scintillation vials, dried and counted for radioactivity. When necessary, neomycin (1.0 mM), amiloride (1.0 mM) and VSCC blokers (verapamil 10^{-6}M, diltiazem 10^{-6}M and nifedipine 10^{-7}M) were added during the last 10 minutes of the preincubation period. $^{45}Ca^{2+}$ influx was expressed as nmoles Ca^{2+}/mg protein, and represents the net influx of Ca^{2+} (total influx minus blank value).

$^{45}Ca^{2+}$ efflux. $^{45}Ca^{2+}$ efflux was measured by a modification of the superfusion method described for neurotransmitter release (26). Synaptosomes were labeled with $^{45}Ca^{2+}$ (0.5 μCi/ml) during 15 min at 37°C. Aliquots (0.5 ml) of the synaptosomal suspension were gently layered onto (0.65 μm) filters placed at the bottom of a set of parallel superfusion chambers. The synaptosomes were washed with Ringer-Tris medium (pH 7.5) at a flow rate of 0.62ml/min during 10 min. Experiment started by replacing the superfusion medium with Ringer-Tris adjusted to different pH values (from 7.5 to 6.5). Fractions were collected at 1 min intervals during 12 min. When the effect of amiloride or neomycin was tested, the Ringer-Tris medium was supplemented with the appropriate agent. Fractions were collected at 1min interval during 12min. Filters and fractions collected were counted for radioactivity. Results are expressed as percentage of total radiactivity (released plus remaining on filters).

Phosphoinositide hydrolysis determination. Phosphoinositide hydrolysis was determined as described by Claro and co-workers (27). Rat cerebral cortical slices were prepared and incubated in Krebs-Henseleit buffer (116 mM NaCl, 1.2 mM KH_2PO_4, 4.7 mM KCl, 1.2 mM Mg_2SO_4, 11 mM glucose, 25 mM $NaHCO_3$ equilibrated with 95% O_2/5% CO_2) for 30 min at 37°C.

Aliquots were incubated with Krebs-Henseleit buffer supplemented with [^{3}H]-inositol (2 μCi/ml) in a proportion 1:4 (vol/vol) at 37°C for 2 h. [^{3}H]-inositol loaded slices were washed with the buffer, sedimented, and synaptosomes were isolated as described above. Synaptosomes were then incubated in the same conditions as described for $^{45}Ca^{2+}$ influx. Incubation was stopped with 1.2 ml of chloroform/methanol (1:2 v/v), and 0.5 ml of both chloroform and 0.25 M HCl, were added to separate the two phases. [^{3}H]-inositol phosphates present in the aqueous phases were counted together after separation from [^{3}H]inositol and [^{3}H]glycerolphosphoryl inositol by Dowex chromatography. Samples of the chloroform phases containing [^{3}H]-inositol lipids were also counted to normalize results as a percentage breakdown of [^{3}H]lipids.

Statistics. Data are presented as means ± SEM of at least three independent determinations done in triplicated. The mean values were compared by variance analysis.

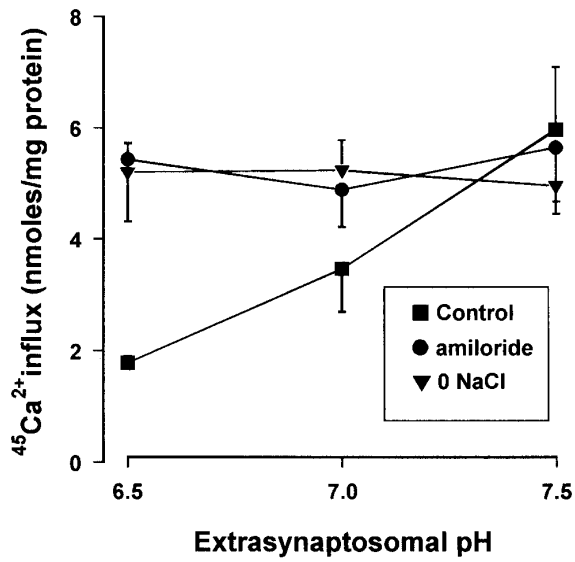

FIG. 1. Effect of pH_e on Ca^{2+} influx. $^{45}Ca^{2+}$ influx was assayed at different pH_e values as is indicated in Material and Methods. Synaptosomes were incubated in Ringer-Tris medium in the presence (■) or in the absence of NaCl (▼) and in the presence of 1.0mM amiloride (●). Data are expressed as means±SEM of six independent experiments done in triplicate. In Ringer-Tris medium two way ANOVA indicates that $^{45}Ca^{2+}$ entry is dependent on the pH_e variation ($p<0.001$).

RESULTS

In order to evaluate the influence of pH_e on Ca^{2+} fluxes, the Ca^{2+} influx and Ca^{2+} efflux were determined in parallel samples. The effect of pH_e (between 7.5-6.5) on Ca^{2+} influx is shown in Fig 1. Ca^{2+} influx was linearly decreased (5 nmol Ca^{2+}/mg protein/pH unit) by external acidification. The decrease in Ca^{2+} entry was already observed one minute after the exposure of synaptosomes to the different pH_e and was maintained for at least 20 min (data not shown). In order to see whether VSCC were involved in the observed decrease in Ca^{2+} influx by extracellular acidification, we have studied the effect of several VSCC blockers. Neither nifedipine (10^{-7} M), nor diltiazem (10^{-6} M) or verapamil (10^{-6} M) were able to block the decrease in Ca^{2+}

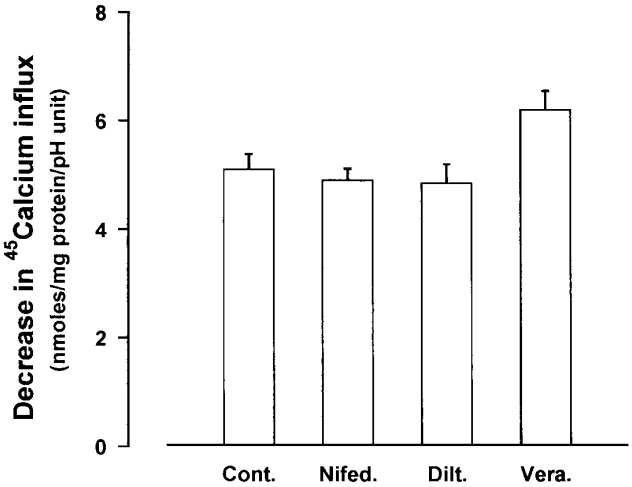

FIG. 2. Effect of Ca^{2+} channel antagonists on Ca^{2+} influx. Ca^{2+} influx was assayed in $^{45}Ca^{2+}$ loaded synaptosomes preincubated for 10 minutes in Ringer-Tris medium (cont.) in the presence of verapamil (10^{-6}M) (vera.), diltiazem (10^{-6}M) (dilt.), or nifedipine (10^{-7}M) (nifed.) at different pH_e values as is described in Material and Methods. Bars indicate the decrease in $^{45}Ca^{2+}$ influx when pH_e was reduced from 7.5 to 6.5. Results are expressed as nmoles/mg protein/pH unit. Note that the decrease in Ca^{2+} entry was not significantly modified by the presence of the VSCC blockers. Results are means±SEM of four independent experiments done in triplicate.

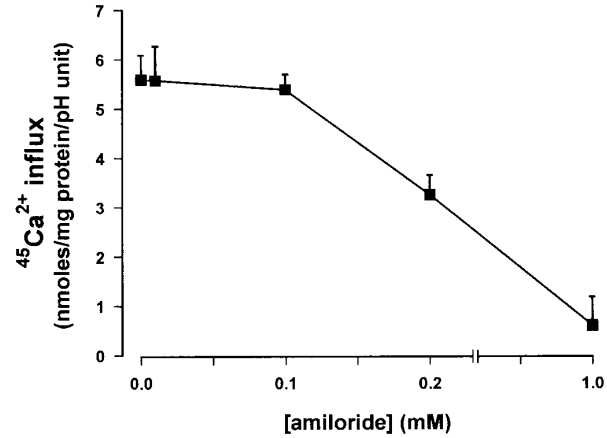

FIG. 4. Concentration-dependence of Ca^{2+} influx inhibition by amiloride. Synaptosomes were preincubated for 10 minutes in the presence of various concentrations of amiloride (0.01-1.0mM). The influx at each concentration of the inhibitor is expressed as nmoles/mg protein/pH unit. Results are means±SEM of four independent experiments performed in triplicate.

entry observed with acidification (Fig.2). On the other hand, Ca^{2+} efflux increased 1.5 fold/pH unit when pH_e was reduced (Fig.3). The rate of the Ca^{2+} release was maximal during the first 3 min and reached a steady-state level after 5 min (data not shown).

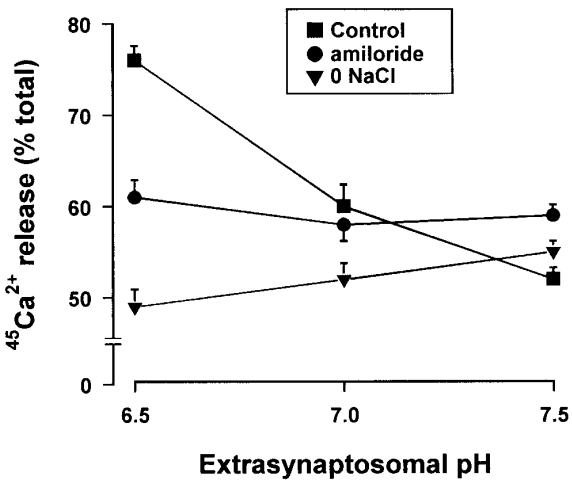

FIG. 3. Effect of pH_e on Ca^{2+} efflux. Ca^{2+} efflux was assayed in $^{45}Ca^{2+}$ loaded synaptosomes perfused with Ringer-Tris medium in the presence (■) or in the absence of NaCl (▼) and in the presence of 1.0mM amiloride (●) at different pH_e values from 7.5 to 6.5. One minute fractions were collected during 12 minutes. Data represent the percentage of $^{45}Ca^{2+}$ release in 5 minutes. Results are means±SEM of six independent experiments performed in triplicate. Two way ANOVA indicates that Ca^{2+} efflux in control conditions is dependent on pH_e variation ($p<0.001$).

Next, we tested whether the changes on Ca^{2+} fluxes induced by a variation in the pH_e were Na^+-dependent. Figures 1 and 3 show that when synaptosomes were incubated with a medium lacking NaCl, Ca^{2+} fluxes did not significantly change when pH_e was decreased from 7.5 to 6.5. In contrast, the Na^+-dependent Ca^{2+} fluxes (determined by the difference between Ca^{2+} fluxes in the presence and in the absence of NaCl) were affected by changes in pH_e. In this respect, it was possible to observe that the Na^+-dependent Ca^{2+} influx progressively decreased and the Na^+-dependent Ca^{2+} efflux progressively increased when pH_e was lowered. These results suggest that a Na^+-dependent mechanism is involved in the changes of synaptosomal Ca^{2+} fluxes observed during the acidification of the extrasynaptosomal medium.

One possible explanation for the variation observed in Ca^{2+} efflux during the acidification is an activation of the Na^+/Ca^{2+} exchanger by protons. Such possibility will explain the increase in Ca^{2+} release and the decrease in Ca^{2+} entry observed when the extracellular medium was acidified. The eventual involvement of the exchanger was examined in the presence of amiloride (a Na^+/Ca^{2+} exchanger blocker). Amiloride was able to inhibit in a concentration-dependent manner the decrease in Ca^{2+} influx produced by acidification (Fig. 4). Maximal inhibition (99%) was observed with 1 mM amiloride. Not only the decrease in Ca^{2+} influx but also the increase in Ca^{2+} efflux was completely inhibited by amiloride (1 mM) (Fig 3). These results suggest that proton activation of the Na^+/Ca^{2+} exchanger could be involved in the observed changes in synaptosomal Ca^{2+} fluxes.

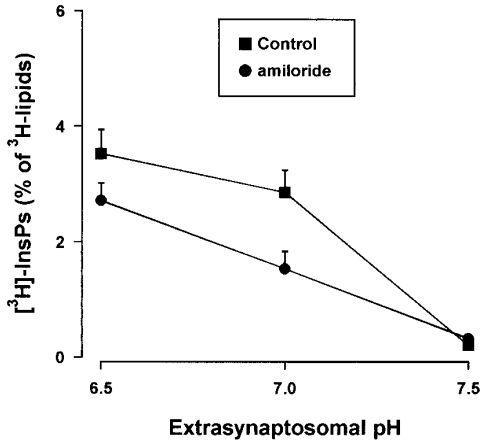

FIG. 5. Effects of pH_e on phosphoinositide hydrolisis. Synaptosomes were isolated from [^{3}H]-loaded slices and then were incubated in the same conditions described above for $^{45}Ca^{2+}$ influx (see Material and Methods). The hydrolisis of phosphoinositides was measured at different pH_e values in Ringer-Tris medium (■) and in the presence of 1.0mM amiloride (●). Two way ANOVA indicates that phosphoinositide hydrolisis is dependent on external pH ($p<0.001$). Data are means±SEM of three independent experiments done in triplicate.

However, we could not know whether this proton activation is a direct or indirect effect. The activation of the Na^+/Ca^{2+} exchanger could result from an increase in the intracellular calcium levels. It is well known that inositol 1,4,5-triphosphate stimulates Ca^{2+} release from intracellular stores. Thus, we studied the eventual effect of a decrease in pH_e on the hydrolisis of phosphoinositides. In R-T medium phosphoinositide breakdown was very low at 7.5. At lower pH_e (7 and 6.5) the phosphoinositide hydrolisis was 3% and 3.5% of [^{3}H]-inositol lipids respectively (Fig 5). Inhibition of the Na^+/Ca^{2+} exchanger by amiloride (1.0 mM) did not significantly modify the hydrolisis of phosphoinositides (Fig 5).

To see whether the hydrolisis of phosphoinositides is involved in the observed changes in Ca^{2+} fluxes during acidification, we inhibited the phospholipase C with neomycin. As it is shown in table 1, extrasynaptosomal acidification neither decreased Ca^{2+} influx nor increased Ca^{2+} efflux in the presence of neomycin 1.0mM indicating an involvement of phospholipase C activity.

DISCUSSION

The intracellular Ca^{2+} concentration in the presynaptic terminal is a key parameter to modulate neurotransmitter release. However, no much data exist about the effect of extracellular acidification on Ca^{2+} homeostasis in the presynaptic terminal. Using a synaptosomal preparation from rat brain we have observed that lowering the pH_e from 7.5 to 6.5 produced a parallel increase in Ca^{2+} efflux and a decrease in Ca^{2+} influx.

In presynaptic terminals, there are different types of VSCC involved in Ca^{2+} entry (17,28) and two major mechanisms for Ca^{2+} extrusion: a Na^+-independent Ca^{2+} efflux mediated by the Ca^{2+}-pump and a Na^+-dependent Ca^{2+}-efflux mediated by the Na^+/Ca^{2+} exchanger (18,19), that could be involved in the observed effects of extracellular acidification on Ca^{2+} fluxes.

It has been previously suggested that synaptosomal VSCC could be a target for protons (16). In this context, several reports have shown that external alkalinization increases the conductance of L-type of VSCC, whereas an opposite effect is evoked by acid pH_e (29,30,31). However, our results indicate that Ca^{2+} channels are not involved in the effects elicited by pH_e on Ca^{2+} fluxes, since the Ca^{2+} channels blockers, diltiazem, verapamil and nifedipine did not alter the effect of pH_e on Ca^{2+} entry (Fig.2). Moreover, verapamil did not modified the effects of pH_e on Ca^{2+} efflux (data not shown).

While we could discard that VSCC are the molecular targets for the observed effects of protons on Ca^{2+} fluxes, the possibility exists for a participation of the mechanisms involved in Ca^{2+} extrusion from synaptic terminals. Our data indicated that the proton effect on Ca^{2+} fluxes was Na^+-dependent. This will suggest that the Ca^{2+}-pump is not involved since it works in a Na^+-independent way. We believe that it is the Na^+/Ca^{2+} exchanger which it is more likely to be involved. This possibility is further supported by the inhibition of the proton effect observed in the presence of the Na^+/Ca^{2+} exchanger inhibitor amiloride (Figs. 1 and 3). Thus, the Na^+/Ca^{2+} exchanger stimulation by acid pH_e will promote an increase in synaptosomal Ca^{2+} efflux and a consequent decrease in global Ca^{2+} influx.

However, we did not know whether the proton stimulation of the exchanger is a direct or an indirect effect.

TABLE 1

Effect of Neomycin on Ca^{2+} Influx and Ca^{2+} Efflux in Rat Brain Synaptosomes at Different pH_e

	Calcium influx		Calcium efflux	
pH	Basal	1 mM neomycin	Basal	1 mM neomycin
7.5	5.97 ± 1.13	5.24 ± 0.49	50 ± 1.15	55 ± 1.15
7.0	3.47 ± 0.77	5.69 ± 0.28	60 ± 1.32	54 ± 1.18
6.5	1.78 ± 0.12	5.57 ± 0.25	78 ± 1.26	49 ± 1.90

Note. Isolated synaptosomes were preincubated for 10 minutes at the indicated pH_e in the presence of 1.0 mM neomycin. Ca^{2+} influx was determined after loading synaptosomes with $^{45}Ca^{2+}$ for 1 minute. Results are expressed as nmoles/mg protein. $^{45}Ca^{2+}$ efflux was determined by collecting 1 minute fractions during 12 minutes from loaded synaptosomes as described in Material and Methods. Results are expressed as the percentage of $^{45}Ca^{2+}$ release. Neither Ca^{2+} infflux nor Ca^{2+} efflux was affected by external acidification in the presence of neomycin ($p < 0.001$, two way ANOVA). Data represent means ± SEM of three independent experiments done in triplicate.

Several studies on non-neuronal and neuronal cells have shown that a reduction in pH_e was associated with a rise in intracellular Ca^{2+} free concentration, probably by mobilizing Ca^{2+} stored in intracellular organelles (32). For example, acid pH_e produces a progressive increase in intracellular Ca^{2+} concentration in fibroblasts which is mediated by phosphoinositide breakdown (6). Our results seem to indicate that a similar mechanism is involved in the observed effect of pH_e on synaptosomal Ca^{2+} fluxes. First, we have observed that low pH_e stimulates phosphoinositide hydrolysis (Fig. 5) and second, inhibition of phospholipase C with neomycin did inhibit the effect of acidic pH_e on Ca^{2+} fluxes (table 1). Moreover, the fact that the increase in phosphoinositide hydrolysis observed in acidic conditions is amiloride-insensitive, suggests that the first target for protons is probably phospholipase C as suggested by other authors (6).

In conclusion, we suggest that external acidification is able to stimulate phospholipase C in synaptic terminal, leading to an increase in phosphoinosite hydrolysis and Ca^{2+} mobilization. The increase in intracellular Ca^{2+} concentration would stimulate the Na^+/Ca^{2+} exchanger and cause an increase in the Ca^{2+} efflux.

This mechanism will explain how extracellular acidification could decrease neurotransmitter release. In this context, it has been described that dopamine release from brain synaptosomes is reduced when pH_e decreases (5). Our results will show that a reduction in neurotransmitter release by extracellular acidification will be caused by a decrease in the intracellular Ca^{2+} levels in the presynaptic terminal by activation of the Na^+/Ca^{2+} exchanger. The activation of the exchanger by protons could be a neuroprotective factor in cerebral ischemia, where neuronal damage is believed to be caused by an increase in glutamate release and an overstimulation of the NMDA type of glutamate receptors (10,15,33). In this respect, several studies have shown that extracellular acidosis (pH_e=6.5) markedly attenuated glutamate neurotoxicity mediated by NMDA receptor (14,15). It is then possible that extracellular acidosis could be neuroprotective by activating the Na^+/Ca^{2+} exchanger, decreasing the Ca^{2+} concentration and reducing the release of glutamate from synaptic terminals. Experiments are currently underway in the laboratory to evaluate this possibility.

ACKNOWLEDGMENT

This work was supported by a research grant from the Fondo de Investigaciones Sanitarias.

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