Effects of Lowering Extracellular and Cytosolic pH on Calcium Fluxes, Cytosolic Calcium Levels, and Transmitter Release in Presynaptic Nerve Terminals Isolated from Rat Brain

PIERRE DRAPEAU and DANIEL A. NACHSHEN†

From the Neurosciences Unit, Montreal General Hospital Research Institute and McGill University, Montreal, Quebec H3G 1A4, Canada, and the Department of Physiology, Cornell University Medical College, New York, New York 10021

ABSTRACT We examined the effects of extracellular and intracellular pH changes on the influx of radioactive 45Ca, the concentration of ionized Ca (pCa) as monitored with the Ca-sensitive fluorescent indicator fura-2, and the efflux of dopamine in presynaptic nerve endings (synaptosomes) isolated from rat brain corpora striata and preloaded with [3H]dopamine. Cytosolic pH (pHi) was monitored by loading the synaptosomes with the H+-sensitive fluorescent indicator 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) (see Nachshen, D. A., and P. Drapeau, 1988, Journal of General Physiology, 91:289-303). An abrupt decrease of the pH of the external medium, from 7.4 to 5.5, produced a slow decrease of pHi (over a 5-min period) from an initial value of 7.2 to a steady state level of ~5.8. When 20 mM acetate was present in acidic media, pH dropped as fast as could be measured (within 2 s) to a level similar to that reached (more slowly) in the absence of acetate. It was therefore possible to lower pH over short time periods to different levels depending on whether or not acetate was present upon extracellular acidification. Extracellular acidification to pH 5.5 (in the absence of acetate) had no significant effect on pCa and dopamine release over a 30-s period (pHi = 6.4). Acidification in the presence of acetate lowered pH, to 5.8 without affecting pCa, but dopamine efflux increased ~20-fold. This increase in basal dopamine release was also observed in the absence of extracellular Ca. Thus, intraterminal, but not extracellular, acidification could stimulate the efflux of dopamine in a Ca-independent manner. The high Q10 (3.6) of acid-stimulated dopamine efflux in the presence of nomifensine (which blocks the dopamine carrier) was consistent with an activation of vesicular dopamine release by H+. When synaptosomes were both depolarized for 2 s in high-K (77.5 mM) solutions and acidified (in the absence of acetate), there was a parallel block of 45Ca entry and evoked dopamine release (50% block at pH 6.0 with 0.2 mM external Ca). When

† Deceased December 16, 1986.
Address reprint requests to Dr. Pierre Drapeau, Neurology Dept., Montreal General Hospital, Montreal, Quebec H3G 1A4, Canada.
acetate was included in the acidic media to further reduce pHi. Ca entry remained blocked, but evoked dopamine release was increased. Therefore, extracellular, but not cytosolic, acidification inhibited the release of dopamine by blocking voltage-gated Ca channels. The stimulation by cytosolic acidification of both basal and evoked dopamine release suggests that vesicular release in resting and depolarized synaptosomes was directly activated by cytoplasmic H⁺.

I N T R O D U C T I O N

Many cellular processes are controlled by changes in the extracellular or intracellular pH (Busa and Nuccitelli, 1984). In acidic media, the evoked release of transmitter at the frog neuromuscular junction is reduced (del Castillo et al., 1962) because of a diminution of the number of quanta liberated by the nerve impulse (Landau and Nachshen, 1975). In contrast, the spontaneous release of transmitter at the frog (Landau and Nachshen, 1975; Cohen and van der Kloot, 1976) and rat neuromuscular junction (Hubbard et al., 1968) is increased by extracellular acidification. The nature of these opposite effects of pH on evoked and spontaneous transmitter release is unknown and is difficult to study because of the small size of the presynaptic terminals.

This question can be examined in isolated presynaptic nerve endings or synaptosomes. This preparation retains many physiological properties of intact terminals (Bradford, 1975; Blaustein et al., 1977), including a tight coupling of transmitter release to Ca influx during brief depolarization (Drapeau and Blaustein, 1983). More importantly, both the cytosolic ionized Ca level (pCa) (Meladolesi et al., 1984; Richards et al., 1984; Nachshen, 1985a) and cytosolic pH (pH) (Nachshen and Drapeau, 1988) can be measured in synaptosomes using ion-selective intracellular fluorescent indicators. It was thus possible to examine the effects of lowering extracellular pH (pH) and pH, on Ca fluxes, pCa, and transmitter (dopamine) release in synaptosomes.

A brief report of these results has been presented elsewhere (Nachshen and Drapeau, 1986).

M E T H O D S

Preparation of Synaptosomes

Synaptosomes were prepared as described in the accompanying article (Nachshen and Drapeau, 1988) from the corpora striata dissected from rat brains, since these are enriched in dopaminergic endings and can be used to study dopamine release (Drapeau and Blaustein, 1983).

Determination of pH and pCa

pH, was measured as described in the accompanying article (Nachshen and Drapeau, 1988) using the fluorescent pH indicator 2',7'bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF).

The free cytosolic Ca concentration (Ca) in synaptosomes was determined using the fluorescent Ca indicator fura-2. In brief, 5 μM of the acetoxymethyl ester derivative of fura-2 (fura-2/AM; Molecular Probes, Inc.) was added to the resuspended synaptosomes. After 30 min at 30°C, the suspension was diluted with 20 vol of low-K solution containing (in millimolar): 5 KCl, 145 NaCl, 1 MgCl₂, 10 2-(N-morpholino)ethane sulfonic acid
The synaptosomes were centrifuged and resuspended in low-K solution and kept at 30°C. For fluorescence measurements, 1 vol of synaptosomes was diluted 20-fold in low-K solution and centrifuged for 45 s in a centrifuge (Microfuge, Beckman Instruments, Inc., Fullerton, CA). The pellet was resuspended in low-K solution and added to 20 vol of low-K solution in a quartz cuvette.

Fluorescence was measured with a Farrand System 3 (Valhalla, NY) spectrofluorometer. Excitation and emission wavelengths of 339 nm (2-nm slit) and 520 nm (10-nm slit) were employed. The emission light was filtered with a cutoff filter (50% transmission at 506 nm). The sample temperature was maintained constant by a water jacket around the cuvette holder, and the sample was stirred with a magnetic stirrer.

The cytosolic Ca concentration ([Ca]) is related to fluorescence intensity (F) by (Rink et al., 1982):

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[Ca] = K_d \times \frac{(F - F_{min})}{(F_{max} - F)},
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where \(F_{min}\) is the Ca-independent fluorescence, measured with \([Ca] \leq 1\) mM, \(F_{max}\) is the maximal fura-2 fluorescence, measured with \([Ca] \geq 1\) mM, and \(K_d\) is the apparent fura-2-Ca dissociation constant.

![Graph showing determination of the Ca-fura-2 dissociation constant (Kd) at pH levels of 7.05 and 6.05. The fluorescence (F) of fura-2 (1 µM) was measured in solutions containing varying amounts of free Ca ([Ca²⁺]), with the pH adjusted to either 7.05 (triangles) or 6.05 (circles). The composition of the solutions and the calculation of [Ca²⁺] is described in the Methods. Measurements have been normalized to the maximal fluorescence level (\(F_{max}\), obtained in solutions with a free Ca concentration of 20 µM, minus the Ca-independent fluorescence, obtained in nominally Ca-free solutions). The curves have been drawn to the equation \(F = F_{max}/(1 + K_d/[Ca])\), and have been fitted to the data points by eye. The arrows indicate the \(K_d\) values that were used to obtain the two curves.]

The \(K_d\) values for Ca–fura-2 were determined from fluorescence measurements in solutions that contained 1 M fura-2. The free Ca concentration was adjusted by the addition of CaCl₂ and the pH was adjusted by the addition of Tris or HCl. The free Ca concentration was calculated using effective Ca-EGTA dissociation constants of 267 nM (pH 7.05), 13.2 µM (pH 6.2), and 26.2 µM (pH 6.05), and Mg-EGTA dissociation constants of 9.1 mM (pH 7.05), 71 mM (pH 6.2), and 101 mM (pH 6.05). These values were calculated from the absolute stability and enthalpy constants tabulated in Martell and Smith (1974). The \(K_d\) values for Ca–fura-2 were 200 nM at pH 7.05 (Fig. 1, triangles), 205 nM at pH 6.2 (not shown), and 280 nM at pH 6.05 (Fig. 1, circles). Adjusting the pH from 7.05 to 6.05 had no effect on the maximal fluorescence level, obtained in solutions with 20 µM (saturating) free Ca, or on the Ca-independent fluorescence level, obtained
in (nominally) Ca-free solutions. After the fluorescence of a fura-2-loaded synaptosome sample was measured, 10 mM EGTA was added to the cuvette, along with sufficient Tris to raise the external pH to 9.2 or more. This addition caused an immediate drop in the fluorescence signal owing to the stripping of Ca from any extracellular fura-2 and enabled us to estimate the fura-2 leak. In general, this leak amounted to no more than 4–8% of the total fura-2 in the sample. Next, 100 μM of digitonin was added to permeabilize the synaptosomal plasma membrane, thereby allowing $F_{\text{min}}$ to be determined. Finally, 1 mM Ca was added, to saturate the fura-2 and allow $F_{\text{max}}$ to be estimated.

In all experiments, synaptosome autofluorescence was determined with control, "non-loaded" synaptosomes that were prepared in parallel with the fura-2-loaded batch and the value was subtracted from those obtained with fura-2.

Measurement of $^{45}\text{Ca}$ Entry
$^{45}\text{Ca}$ entry was determined as described by Nachshen and Blaustein (1980). Aliquots of the synaptosome suspension were added to equal volumes of a low-K (5 mM K, 145 mM Na) or K-rich (77.5 mM K, 72.5 mM Na) solution that contained the tracer. Some solutions (as indicated) contained 20 mM acetate and the pH was adjusted to the values shown. $^{45}\text{Ca}$ entry was terminated and the $^{45}\text{Ca}$ content was determined as described in the accompanying article (Nachshen and Drapeau, 1988). K-stimulated $^{45}\text{Ca}$ entry was calculated as the difference between $^{45}\text{Ca}$ uptake from the low-K solution and from the K-rich solution.

Determination of Dopamine Release
Synaptosomes were labeled with radioactive dopamine by incubation for 30 min at 30℃ in low-K solution containing 0.3 μM $[^{3}H]$dopamine (100 Ci/mm mol), 0.5 mM ascorbate (to prevent oxidation), and 0.05 mM pargyline (a monoamine oxidase inhibitor). An aliquot of the labeled fraction (50 μl, containing ~50 mg protein) was pipetted into one well of a filtration tank (VFM-2, Amicon Corp., Danvers, MA) containing a glass-fiber filter as described previously (Drapeau and Blaustein, 1983) and 1.5 ml of low-K solution containing 0.1% fatty acid-free bovine serum albumin (BSA) (to reduce lysis of the synaptosomes). The synaptosomes were washed free of the unincorporated tracer by five repeated additions of low-K solution containing BSA, followed by suction. Transmitter release was then initiated by adding either a low-K or K-rich solution containing 0.1% BSA and 1 mM nomifensine (to block the dopamine carrier) adjusted to the pH indicated in the text. In experiments where high-K test solutions containing acetate were used to evoke dopamine release (as indicated), the test solutions were added 2 s after a low-K solution containing acetate was added in order to allow sufficient time for intracellular acidification (see text). Release was terminated 2 or 30 s later by adding ice-cold low-K solution containing 2 mM CdCl₂ (to block Ca channels) and applying a vacuum. The collected effluent and the filter pads were assayed for radioactivity by liquid scintillation spectrometry.

RESULTS

Relationship between $pH_o$ and $pH_i$

The effect of extracellular acidification on $pH_i$ is shown in Fig. 2. In the absence of acetate, when $pH_o$ was lowered from 7.4 to 5.5, there was an "instantaneous" decrease in BCECF fluorescence followed by a much slower drop (Fig. 2A). The initial fluorescence drop was mostly due to titration of extracellular BCECF that
had leaked out of the synaptosomes. This could be seen in experiments where the synaptosomes were pelleted and the fluorescence of the supernatant decreased to a similar extent (see the inset to Fig. 2A). The slow fluorescence decrease is due to cytosolic acidification (see Nachshen and Drapeau, 1988). A decrease in pH_in, from 7.4 to 5.5, resulted in a drop in fluorescence corresponding to cytosolic acidification from a pH_in of 7.2 to ~6.4 after 30 s and leveled off at ~5.8 within 3 min. (pH_in was estimated from steady state fluorescence measurements of permeabilized synaptosomes and is corrected for the fluorescence of extracellular dye; see Nachshen and Drapeau, 1988).

When both HCl and 20 mM acetate were added to the synaptosome suspension, there was an immediate acidification of pH_in (Fig. 2B) to a level similar to the final level observed in the absence of acetate. The fluorescence decrease was as fast as could be measured (complete within 2 s). The increased rate of cytosolic

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**Figure 2.** Effect of extracellular acidification and acetate on pH_in. The fluorescence of BCECF-loaded synaptosomes was measured in medium at pH 7.4. At the time indicated by the arrows, sufficient HCl without (A) or with (B) 20 mM acetate was added to lower pH_in to 5.5. The inset to A shows the fluorescence change of extracellular dye when HCl was added to the synaptosome-free supernatant obtained after centrifugation of the suspension.

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**Figure 3.** pH dependence of basal dopamine release. Dopamine release was measured at 2 and 30 s after addition of low-K solutions without (open bars) or with (filled bars) 0.2 mM Ca present at the pH indicated. The pair of bars on the right represents data obtained at pH 5.5 when 20 mM acetate was included in the test solutions. The percentage of the total dopamine released during the 2-s incubation was subtracted from the data obtained after 30 s and the rate of release was calculated by dividing this value by 28 s, i.e., percent release per second. All measurements were performed in quintuplicate.
acidification results from acetic acid entering the nerve terminals to release H⁺ (see Thomas, 1984). It was therefore possible to lower pH, over short (30 s) time periods to different levels depending on whether or not acetate was present upon extracellular acidification.

**pH Dependence of the Basal Dopamine Release**

When synaptosomes were incubated at pH 5.5 (in the absence of acetate) for 30 s, the rate of [³H]dopamine efflux appeared to increase slightly compared to the rate at pH 7.4 (Fig. 3). In contrast, when acetate was included in the acidic medium, a 5–53-fold increase in basal dopamine release was observed in five experiments (mean increase of 23-fold). The results of an experiment in which dopamine release over a 30-s period was measured in acetate-containing media at different pH values is shown in Fig. 4. Dopamine release was stimulated when pH~ decreased to ~6.3 and less. Addition of 0.2 mM Ca resulted in no further increase in dopamine release in the absence or presence of acetate (Fig. 3). Thus, it appears that cytosolic and not extracellular acidification resulted in a large stimulation of basal dopamine release that was independent of extracellular Ca.

**FIGURE 4.** Effect of varying pH~ and acetate on basal dopamine release. Dopamine release was measured 30 s after addition of low-K solutions without acetate at pH 7.4 or with 20 mM acetate at pH 7.0, 6.5, 6.0, and 5.5. The pH~ values were determined as described in the Methods during parallel measurements from the same batch of synaptosomes. All solutions contained 0.2 mM Ca.

**FIGURE 5.** Effect of acidification on the cytosolic Ca level. The fluorescence of fura-2-loaded synaptosomes (top traces) was measured in low-K medium at pH 7.4. At the time indicated by the first arrow, HCl was added to decrease the pH to 6.0 (left-hand trace). In a subsequent sample, 50 nM ionomycin and then 25 mM KCl were added. The cytosolic Ca scale on the right was determined as described in the Methods. The bottom trace shows the fluorescence of BCECF-loaded synaptosomes. At the first arrow, HCl was added as in the trace above. All solutions contained 0.2 mM Ca.
To determine whether a decrease of pH, could cause a decrease in pCa, we monitored cytosolic Ca with fura-2 (Grynkiewicz et al., 1985). Extracellular acidification to pH 6.0 (Fig. 5) or 5.5 in the presence of acetate (not shown) resulted in no change in fura-2 fluorescence even after several minutes of exposure. (Because of the increased $K_d$ for Ca-fura-2 at pH 6.0, there may be an increase in $C_{a_i}$ of ~40%.) In contrast, a moderate depolarization of the synaptosomes by raising the K concentration to 25 mM, or by addition of ionomycin (50 nM), resulted in a readily detected increase in cytosolic Ca (Fig. 5, right).

Catecholamine accumulation in the storage vesicles depends upon the $H^+$ gradient across the vesicular membrane (Johnson et al., 1982). Cytosolic acidification could collapse this gradient, thereby increasing the free dopamine concentration in the cytosol. Although the dopamine carrier was blocked by the high nomifensine concentration used in our experiments (see Hunt et al., 1974; Raiteri et al., 1978), lowering pH, could potentially increase dopamine release by increasing the rate of passive diffusion of dopamine out of the nerve terminals. Diffusional processes should not be very sensitive to changes in temperature ($Q_{10}$ ~ 1.3) and we examined the temperature dependence of basal dopamine release. The extent and time course of cytosolic acidification (in the presence of acetate) were unaltered when the temperature was lowered from 30 to 3°C for at least 30 s (not shown). Dopamine efflux over a 30-s period in medium at pH 7.4 was similarly not very sensitive to a change in temperature from 30 to 3°C (Fig. 6, filled symbols). Dopamine release evoked by acidification was, however, very sensitive to changes in temperature (Fig. 6, open symbols); e.g., release decreased by 80% when the temperature was lowered from 30 to 20°C ($Q_{10} = 3.6$). It is thus unlikely that the increase in dopamine release evoked by cytosolic acidification resulted from increased passive leak of dopamine from the nerve terminals.

**pH Dependence of Evoked Dopamine Release**

When synaptosomes are briefly depolarized by high-K solution, dopamine release is greatly stimulated (Blaustein and Goldring, 1975). This release is tightly
coupled to an influx of Ca (Drapeau and Blaustein, 1983) through voltage-gated Ca channels (Nachshen and Blaustein, 1980; Nachshen, 1984, 1985b). When pH was lowered, both the high-K-stimulated dopamine release and Ca influx decreased in parallel (Fig. 7). The block of Ca influx and dopamine release by low pH could be explained by an H+ inhibition of Ca channels that have an apparent pKₐ of 6.0 and an apparent Ca dissociation constant of ~200 μM (solid line in Fig. 7), as shown previously for the pH-dependent block of Ca channels (Nachshen and Blaustein, 1979).

When acetate was added to the acidic (pH 5.9) media, the block of dopamine release was partially reversed (see Fig. 8), but the inhibition of Ca influx was
unaffected (not shown). Thus, as with basal dopamine release, evoked release was enhanced by cytosolic acidification.

DISCUSSION

Our observations that cytosolic acidification stimulated basal dopamine release from rat brain synaptosomes and that lowering pH, inhibited evoked release are consistent with previous observations in the frog (del Castillo et al., 1962; Landau and Nachshen, 1975; Cohen and van der Kloot, 1976) and the rat neuromuscular junction (Hubbard et al., 1968). In particular, the requirement for cytosolic, and not just extracellular, acidification for stimulation of basal release agrees with the finding of Cohen and van der Kloot (1976). They showed that exposure of the nerve terminals to elevated pCO₂ and, presumably, cytosolic acidification was much more effective in raising the miniature endplate potential frequency at the frog neuromuscular junction than was comparable acidification of the medium. Our observation that stimulation of basal release by low pH did not require external Ca agrees with the study of the rat neuromuscular junction by Hubbard et al. (1968) but not with the experiments performed on the frog neuromuscular synapse (Cohen and van der Kloot, 1976), where Ca was required for the stimulation.

We did not detect a change in Ca, when pH, was >6.0. At a pH of 6.0, cytosolic Ca increased at most 40%. Raising the external Ca concentration from 0 to 2 mM triples both cytosolic Ca (Fig. 7 of Nachshen, 1985a) and dopamine release (Fig. 4 of Drapeau and Blaustein, 1983). Similarly, raising the K concentration from 5 to 25 mM resulted in a readily detected increase in cytosolic Ca (Fig. 5) and doubled dopamine release (Fig. 5 of Drapeau and Blaustein, 1983). We should therefore have observed a large rise in cytosolic Ca if the ~20-fold stimulation of dopamine release by low pH, was caused by the displacement of bound intraterminal Ca. Our findings support a direct stimulatory role for cytosolic H⁺ during basal dopamine efflux from synaptosomes.

The block of evoked dopamine release that we observed during extracellular acidification could be entirely accounted for by an H⁺ block of voltage-gated Ca channels (Nachshen and Blaustein, 1979; Umbach, 1982). As with basal release, evoked dopamine release was increased by lowering pH. The high temperature sensitivity (Q₁₀ = 3.6) of acid-stimulated basal dopamine release observed in the presence of the dopamine-carrier blocker nomifensine resembled the temperature dependence of spontaneous quantal transmitter release observed at the frog neuromuscular junction (Q₁₀ = 3; Fatt and Katz, 1952). Thus, it seems likely that a common, perhaps vesicular, mechanism of release (see Fried and Blaustein, 1978) is accelerated by cytosolic acidification in both resting and depolarized synaptosomes.

Dopamine release increases 40-fold when synaptosomes are depolarized by K-rich solutions in the presence of extracellular Ca (Drapeau and Blaustein, 1983). Under these conditions, pCa, falls from a resting value of ~7 (Nachshen, 1985a; Meldolesi et al., 1984; Richards et al., 1984) to at least 6.3 (Nachshen, 1985a). Similarly, dopamine release increased ~20-fold under conditions where pH, decreased from 7.0 to 6.1 (Figs. 3 and 4). In this range of concentrations, changes in pCa, and pH, appear to be equipotent in promoting dopamine release. A
simple model for the stimulation of release at low pH, would be the titration of 
a molecule that limits the rate of release, possibly the same molecule activated 
by Ca.

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