

## Demonstration of an $\text{Na}^+/\text{H}^+$ exchanger in mouse keratinocytes measured by the novel pH-sensitive fluorochrome SNARF-calcein

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*(Received 8 December 1996; revision accepted 12 November 1997)*

**Abstract.** In many cell types cytoplasmic alkalization is an early marker for cell activation. An amiloride-sensitive  $\text{Na}^+/\text{H}^+$  exchanger is an important regulator of this process. However, in keratinocytes the existence of a  $\text{Na}^+/\text{H}^+$  exchanger nor a proliferation-associated increase in intracellular pH ( $\text{pH}_i$ ) has been demonstrated.

The aim of this study was to investigate whether or not keratinocytes, derived from the BALB/MK cell line, contain a  $\text{Na}^+/\text{H}^+$  exchanger and whether cytoplasmic alkalization is proliferation-associated in these cells. This mouse keratinocyte cell line can easily be switched between a proliferative and a quiescent state under defined culture conditions. The novel pH-sensitive dye seminaphthorhodafluor (SNARF)-calcein proved to be very suitable for flow cytometric  $\text{pH}_i$  measurements in BALB/MK cells. Initial measurements of the  $\text{pH}_i$  using a cocktail of the established fluorochromes 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) and SNARF-1 failed because of the differential uptake and binding kinetics of these pH-sensitive dyes.

Using SNARF-calcein we were able to show proliferation to be associated with increased  $\text{pH}_i$ . However, culture conditions were critical for these measurements. Our data indicate that the  $\text{Na}^+/\text{H}^+$  exchanger is involved in this process, since acid load and  $\text{pH}_i$ -recovery experiments showed the alkalization to be amiloride-sensitive.

### INTRODUCTION

Epidermopoiesis is the process of continuously renewing epithelial cells in skin. This process has been disturbed in psoriasis, a common chronic hyperproliferative skin disease. In psoriasis one of the hallmarks is an increased recruitment of quiescent ( $G_0$ ) cells (van Erp, Boezeman & Brons 1996). It has been shown in some cells that an alkalization of the cytoplasm occurs at the transition of cells from the  $G_0$  to the  $G_1$  phase *in vitro* (Pouyssegur *et al.* 1985, Musgrove, Seaman & Hedley 1987). We investigated the correlation between changes in  $\text{pH}_i$  and growth status in cultured keratinocytes. It is believed that a membrane  $\text{Na}^+/\text{H}^+$  exchanger, present in almost all vertebrate cells, plays an important role (Noel &

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Pouyssegur 1995). However, the existence of such an enzyme system in keratinocyte plasma membranes has not been shown previously. A commonly used technique for studying the presence of the  $\text{Na}^+/\text{H}^+$  exchanger in the plasma membrane is the acid load and  $\text{pH}_i$ -recovery experiment (Moolenaar, Tertoolen & de Laat 1984), also known as the  $\text{NH}_4^+$ -prepulse technique (Frelin *et al.* 1988). The  $\text{pH}_i$  can be manipulated experimentally by preloading the cell with  $\text{NH}_4^+$ , by other weak acid, or by using ionophores (Thomas *et al.* 1979, Grinstein & Rothstein 1986, van Erp *et al.* 1991). Under  $\text{Na}^+$  and  $\text{HCO}_3^-$  free conditions, the cytoplasmic pH is expected to drop and recover after the addition of  $\text{Na}^+$  ions. If a  $\text{Na}^+/\text{H}^+$  exchanger is responsible for the recovery it can be inhibited by the diuretic amiloride (Seifter & Aronson 1986, Rijzewijk *et al.* 1988).

This approach was applied to an *in vitro* model using the BALB/MK cell line (Weissman & Aaronson 1983). This epidermal growth factor (EGF) dependent mouse keratinocyte cell line was characterized cell kinetically in detail by our group (van Hooijdonk *et al.* 1993). Grown in a low-calcium medium without EGF and serum, the BALB/MK cells can be maintained in a quiescent state. Subsequent restimulation with EGF or serum resulted in a synchronized cohort of cells being recruited (van Hooijdonk *et al.* 1993). This detailed characterization makes the BALB/MK cell line a suitable model for studying proliferation.

For  $\text{pH}_i$  measurements, fluorescent probes such as BCECF and SNARF-1 have been used widely (Valet *et al.* 1981, Musgrove, Rugg & Hedley 1986, Cook & Fox 1988, van Erp *et al.* 1991). Because of differences in metabolic activity and cell size of keratinocytes a ratiometric approach was essential (Valet *et al.* 1981). In the present study we tested the novel pH indicator SNARF-calcein (Slayman *et al.* 1994). We compared this dye with the fluorochromes BCECF and SNARF-1 individually and with a cocktail of the two, using both spectrofluorimetry and flow cytometry. Using the most appropriate dye(s) under optimal conditions, we have tried to answer the following questions: (1) Do BALB/MK cells possess a  $\text{Na}^+/\text{H}^+$  exchanger in their plasma membrane? (2) If a  $\text{Na}^+/\text{H}^+$  exchanger is demonstrated, is cytoplasmic alkalization in these cells proliferation-associated?

## MATERIALS AND METHODS

### Solutions

Stock solutions of 1 mM acetoxy methyl ester of BCECF (BCECF/AM), SNARF-1/AM and SNARF-calcein/AM (all from Molecular Probes Inc., Eugene, OR) were prepared in dimethyl sulphoxide. In general spectrofluorimetric and flow cytometric measurements were carried out with Hank's balanced salt solution (HBSS), without sodium bicarbonate and phenol red/10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer (Sigma, St. Louis, MO). For calibration experiments,  $\text{Na}^+$  and  $\text{K}^+$  buffer solutions were prepared mainly as described earlier (Boyer & Hedley 1994). Stock solutions of 10 mM nigericin (Sigma) in ethanol, 1 mM SNARF-calcein in  $\text{K}^+$  buffer (pH 8.1) and a solution of 2 mM  $\text{NH}_4\text{Cl}$  were prepared. Stock solutions of 2 mM amiloride (Sigma) in  $\text{Na}^+$  buffer and 1 mM iododeoxyuridine (IdUrd) in phosphate-buffered saline (PBS) were stored at  $-20^\circ\text{C}$ .

### Spectrofluorimetry

Fluorescence measurements were performed on a RF-5001PC spectrofluorophotometer (Shimadzu Corp., Kyoto, Japan). The emission spectra were obtained using an excitation wavelength of 488 nm to match the excitation wavelength available from the argon-ion laser of the flow cytometer, while the emission was recorded in the range of 530–650 nm. Emission spectra for a cocktail of BCECF and SNARF-1 were obtained by adding  $0.2 \mu\text{M}$  BCECF and

15  $\mu\text{M}$  SNARF-1 to high  $\text{K}^+$  buffers with pH values from 5.5 to 8 and for SNARF-calcein alone by the addition of 10  $\mu\text{M}$ .

### Cell culture

Media were used and BALB/MK cells were cultured as described previously by our group (van Hooijdonk *et al.* 1993). Briefly, from a basal culture medium, composed of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (1:1, v/v; Ritmeester, Utrecht, The Netherlands), containing 50  $\mu\text{M}$   $\text{CaCl}_2$ , 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin, two working media were prepared. The first, termed 'complete' medium, consisted of basal medium supplemented with 10% dialysed fetal bovine serum (Sigma) and 4 ng/ml EGF (Sigma). The second, termed 'maintenance' medium, was composed of basal medium supplemented with 0.2 mM ethanolamine, 5  $\mu\text{g}/\text{ml}$  transferrin, 10  $\mu\text{g}/\text{ml}$  insulin and 1  $\mu\text{M}$  selenium. In order to obtain exponentially growing keratinocytes, the cells were plated in complete medium and harvested at 70% confluence. For experiments in which cells were required in the quiescent state, the complete medium was removed at 20% to 30% confluence and switched to maintenance medium for 24 or 48 h. All cultures were grown at 37°C in an atmosphere of 8%  $\text{CO}_2$  and 92% relative humidity. The keratinocytes were harvested by washing twice with PBS followed by 10 min trypsinization at 37°C and collected in 8 ml PBS containing 5% fetal calf serum (PBS/FCS).

### Iododeoxyuridine-labelling procedure

The cultures were re-fed with either complete or maintenance medium. About 4 h later the keratinocytes were pulse-labelled for 30 min with 10  $\mu\text{M}$  IdUrd. After harvesting, aliquots of these cells were used for pH<sub>i</sub> measurements, as described below. The remaining part was fixed by resuspending the pellet in 70% ice-cold ethanol, and the suspension was stored at -20°C until further use.

### Immunocytochemical staining of IdUrd

Staining was performed as described previously (van Erp *et al.* 1988, Jensen *et al.* 1994). IdUrd was visualized with Dako-bromodeoxyuridine (Dakopatts, Copenhagen, Denmark) after a one-step combined pepsin/HCl denaturation protocol. Finally, after washing with PBS, the cells were resuspended in 300  $\mu\text{l}$  PBS containing 40  $\mu\text{g}/\text{ml}$  propidium iodide (PI; Calbiochem, San Diego, CA) and 50  $\mu\text{l}$  1% (w/v) RNase A (Sigma) was added. The cells were incubated for 15 min at room temperature and measured on the flow cytometer.

### SNARF-calcein labelling procedure

After centrifugation (400 g, 7.5 min) of the cell suspension in PBS/FCS the pellet was resuspended in 6 ml  $\text{Na}^+$  buffer (pH 7.2). About  $2 \times 10^6$  cells were spun down and resuspended in 1.5 ml 10  $\mu\text{M}$  SNARF-calcein/AM in  $\text{Na}^+$  buffer. After an incubation of 15 min at 37°C, 1 ml cell suspension was spun down, resuspended in 0.5 ml  $\text{Na}^+$  buffer and kept on ice for a maximum of 30 min before measurement. To investigate whether or not the pH<sub>i</sub> measurements were independent of dye load, aliquots of about  $1 \times 10^6$  cells were loaded with 0.5, 1, 2, 5, and 10  $\mu\text{M}$  SNARF-calcein, respectively.

### pH<sub>i</sub> determination

The pH<sub>i</sub> was determined using a ratiometric method, which measures the pH-dependent emission of SNARF-calcein flow cytometrically. The principal of this ratiometric method is basically as described before (van Erp *et al.* 1991). Briefly, the cells were resuspended in  $\text{Na}^+$

buffer and incubated for 30 min at 37°C with 10  $\mu\text{M}$  SNARF-calcein/AM. For each experiment a calibration curve was constructed for the pH range 6.3–8.0 by obtaining the fluorescence ratios of cells. The  $\text{pH}_i$  of the cells was equilibrated to the buffer pH by the addition of the proton ionophore nigericin. Calibration samples were resuspended in  $\text{K}^+$  buffers (pH 6–8) and run in the presence of 10  $\mu\text{M}$  nigericin. In our experiments, where the extra- and intracellular concentration of  $\text{K}^+$  are approximately equal, nigericin allows equilibration of  $\text{pH}_i$  to the external pH (Boyer & Hedley 1994).

### Flow cytometry

All flow cytometric measurements were done with a Epics<sup>®</sup> Elite flow cytometer (Coulter Corporation, Luton, UK) equipped with a 15 mW 488 nm air-cooled argon laser. For  $\text{pH}_i$  measurements, fluorescence emission wavelengths were selected using 525, 575 and 625 nm band pass filters, separated by 550 and 610 nm dichroic filters, respectively. If possible,  $5 \times 10^3$  gated cells were measured. The scatters, the individual fluorescence signals and their ratio (F525/F575 in case of BCECF and SNARF-1 and F625/F575 in case of SNARF-calcein) were stored and analysed.

For measuring FITC, the green fluorescence was detected through a 525 nm band pass filter and for PI a long pass 630 nm filter was used. FITC and PI were separated by a 550 nm dichroic mirror. Usually about  $1 \times 10^4$  cells were measured. The ratio of area–peak discriminates between artefacts due to doublets of diploid cells and real single tetraploid (or late S) cells (Bauer & Boezeman 1983). The data were stored and further analysed.

## RESULTS

In order to improve the resolution of the  $\text{pH}_i$  measurements, initial experiments were carried out using a cocktail of BCECF and SNARF-1. At an excitation wavelength of 488 nm the emission intensity at 525 nm for BCECF strongly increased whereas the emission intensity for SNARF-1 at 575 nm decreased with increasing pH. In this way a ratiometric approach could be applied. When calibration experiments were performed using the nigericin calibration technique, the resolution obtained was not optimal. A separation of 0.2 pH units was not possible. The reason for this unexpected result appeared to be the differential uptake and binding kinetics of BCECF and SNARF-1. This made the cocktail not the first choice for  $\text{pH}_i$  measurements in keratinocytes. In order to obtain this separation, necessary for  $\text{pH}_i$  measurements in keratinocytes, we explored the use of the novel pH indicator SNARF-calcein.

The fluorescence emission spectra (530 nm to 650 nm) of SNARF-calcein in buffers of pH values from 5.5 and 8.0 were shown in Figure 1. The emission maximum of SNARF-calcein shifted from 585 to 610 nm when increasing the pH from 5.5 to 8.0. With increasing pH the emission intensity maximum at 585 nm decreased, whilst the maximum at 610 nm increased. This experiment was performed two times and appeared to be in line with the specifications of the supplier. For flow cytometric measurements band pass filters of 575 and 625 nm were available, so ratiometric measurements were performed at these wavelengths.

Fluorescence ratio histograms (FRHs) for a  $\text{pH}_i$  calibration curve using SNARF-calcein were shown in Figure 2a. The  $\text{pH}_i$  of the keratinocytes was equilibrated to the buffer pH by the addition of nigericin. These FRHs showed a very good resolution over a wide pH range, since peaks differing 0.2 pH units were totally separated at half peak height. The mean values of these histograms were plotted in Figure 2b. From pH 6.3–8.0 this plot yielded a linear curve, fitted using least-squares linear regression analysis. The  $\text{pH}_i$  of the cells in buffer

without nigericin was calculated subsequently from the fitted line. Successive calibrations were performed with every  $\text{pH}_i$ -experiment.

To illustrate that the  $\text{pH}_i$  measurements were independent of dye load, the SNARF-calcein concentration was plotted against the fluorescence intensities at 575 and 625 nm and the fluorescence ratio in Figure 3. The average fluorescence intensity per cell increased with dye content, whereas the fluorescence ratio plotted against the dye concentration showed a horizontal line.

To demonstrate the existence of an  $\text{Na}^+/\text{H}^+$  exchanger in BALB/MK cells, the cells were acid-loaded by the  $\text{NH}_4^+$ -prepulse technique. In brief, before measurement aliquots of about  $1 \times 10^6$  cells were loaded with  $10 \mu\text{M}$  SNARF-calcein for 15 min at  $37^\circ\text{C}$ . Initially, the physiological  $\text{pH}_i$  was measured for 2 min. The mean  $F_{625}/F_{575}$  ratio over the 2-min period was calculated from a calibration curve and appeared to be  $\text{pH}$  7.2. After addition of  $5 \mu\text{l}$  of  $2 \text{ M}$   $\text{NH}_4\text{Cl}$  the  $\text{pH}_i$  immediately rose 0.3  $\text{pH}$  units, followed by a gradual cell acidification (Figure 4, A). After having resuspended the cells in  $\text{K}^+$  buffer the  $\text{pH}_i$  dropped to 6.0 rapidly (B). Resuspending the cells in  $\text{Na}^+$  buffer resulted in a recovery of the  $\text{pH}_i$  to its physiological value within about 10 min (C). Figure 4 showed a typical  $\text{pH}_i$  course in keratinocytes before, during and after a  $\text{NH}_4\text{Cl}$  pulse. The addition of different concentrations of amiloride, before  $\text{Na}^+$ -ions were added, resulted in a dose-dependent inhibition of the  $\text{pH}_i$  recovery (Figure 5). The buffer capacity for cells without amiloride appeared to be 0.087  $\text{pH}/\text{min}$ . The buffer capacity decreased from 0.08  $\text{pH}/\text{min}$  at  $0.1 \mu\text{M}$  amiloride to 0.01  $\text{pH}/\text{min}$  at  $100 \mu\text{M}$  amiloride. This experiment was performed twice.

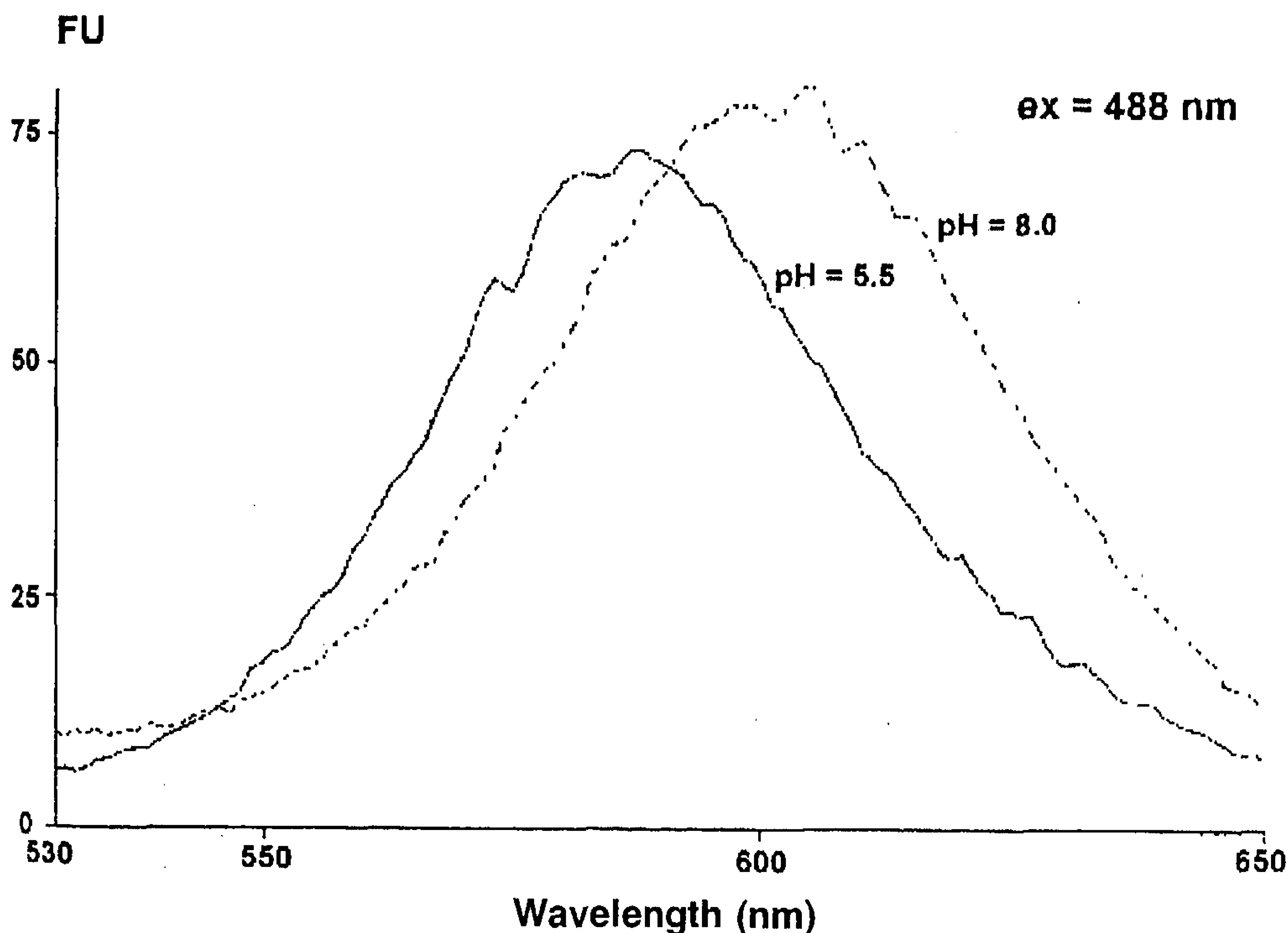
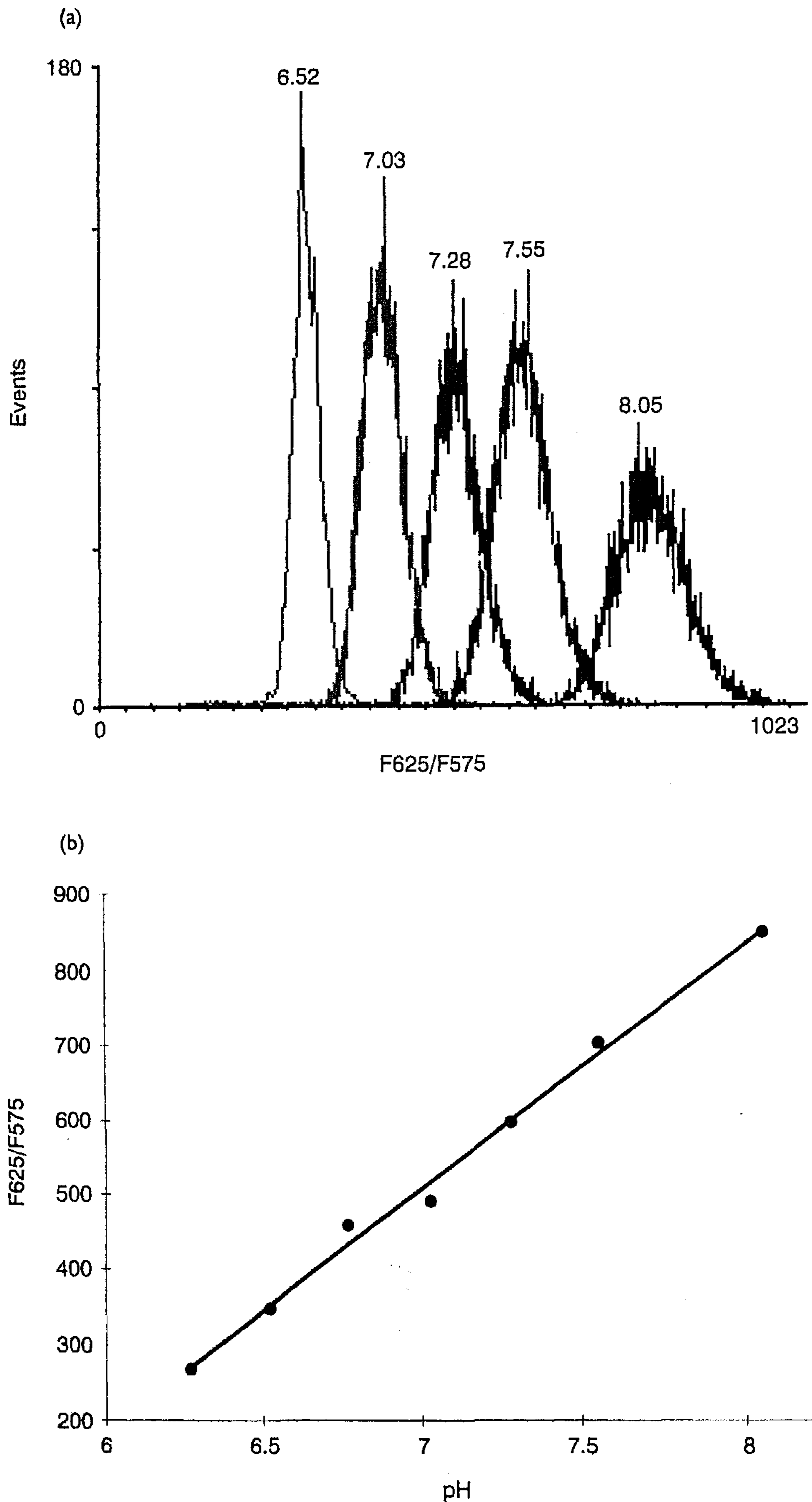
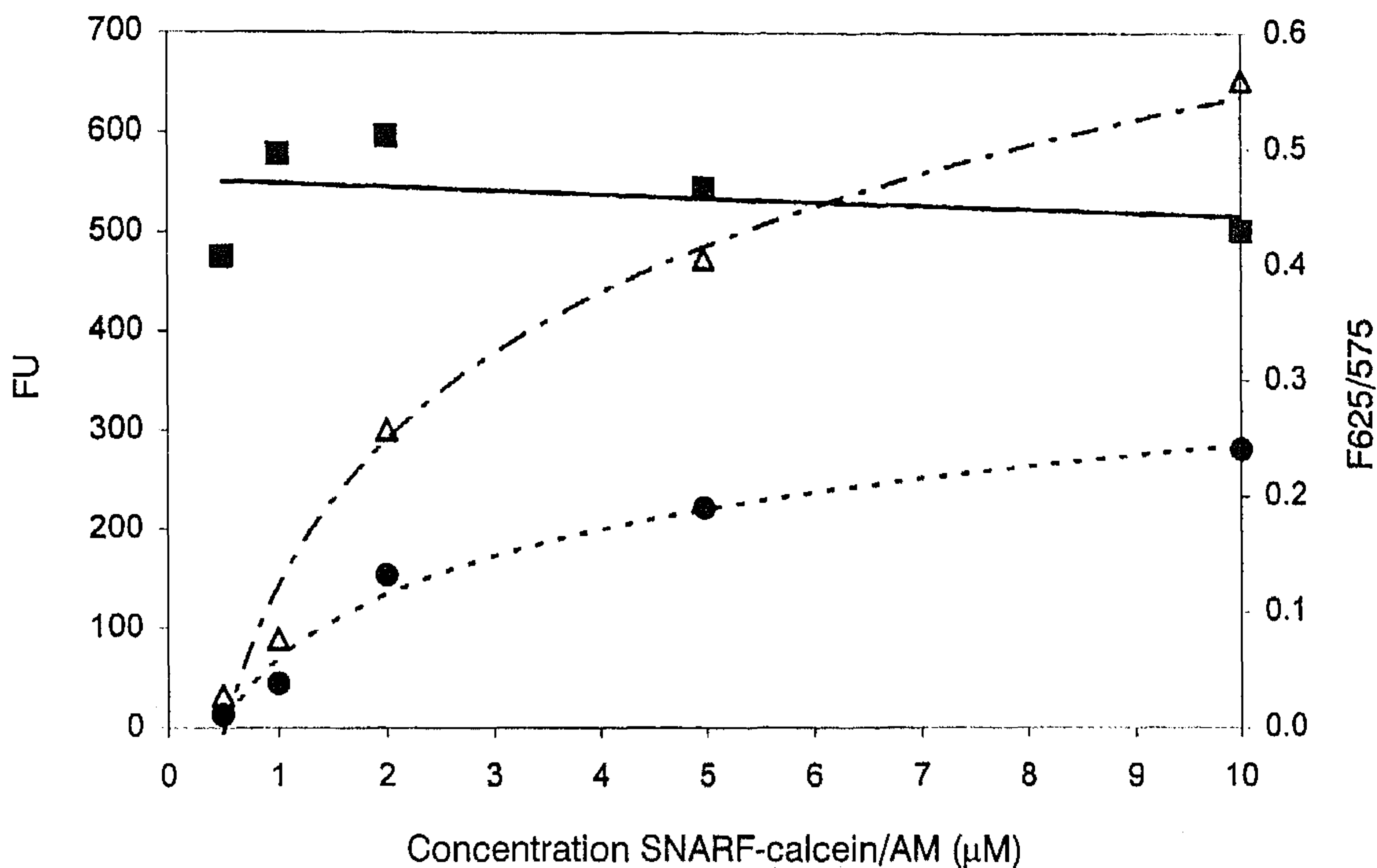


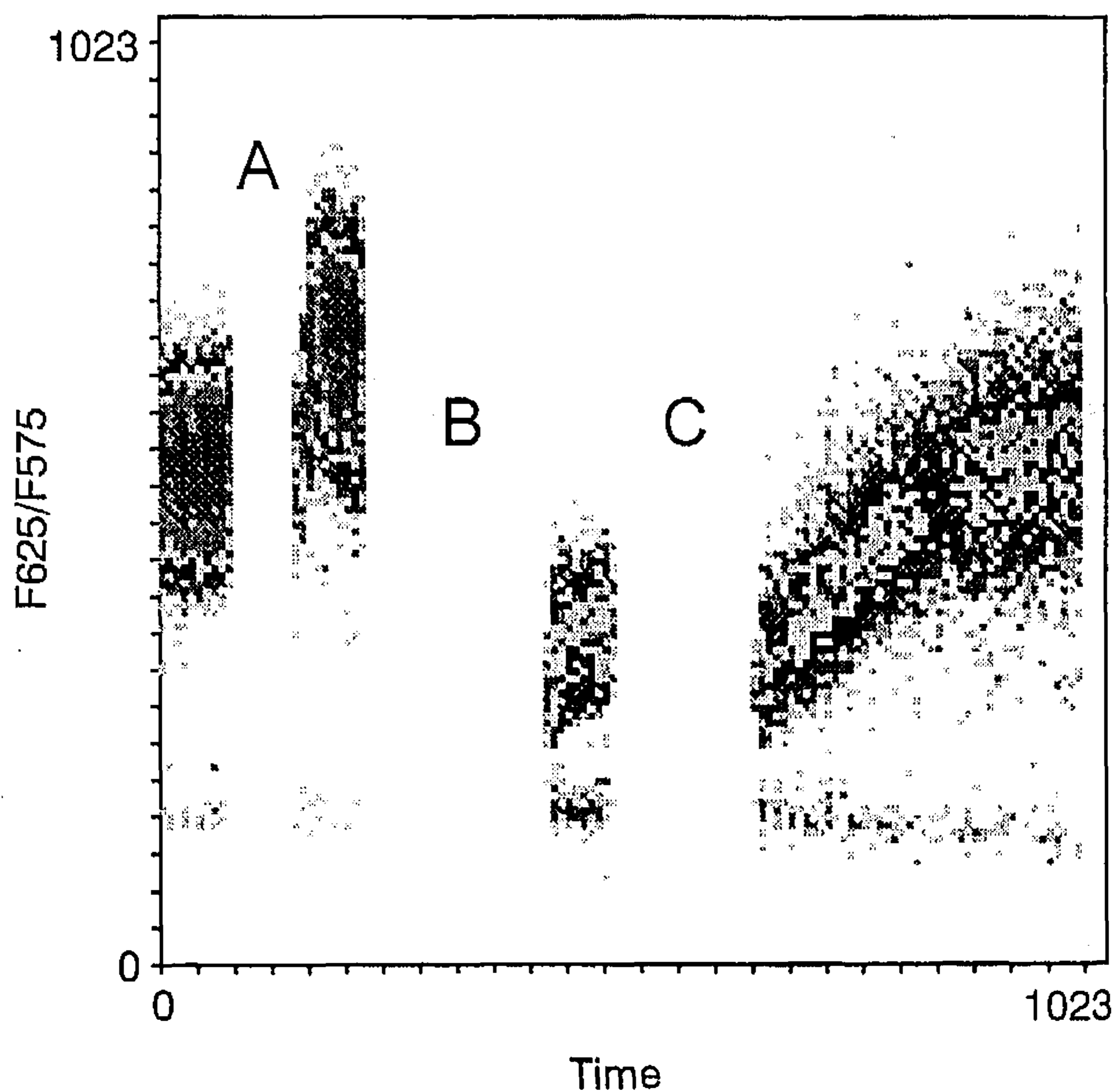
Figure 1. Emission spectra for SNARF-calcein in high  $\text{K}^+$  buffers of  $\text{pH}$  5.5 and  $\text{pH}$  8.0 with an excitation of 488 nm measured spectrofluorimetrically.



**Figure 2.** (a) Flow cytometric measurement of FRHs of keratinocytes loaded with SNARF-calcein/AM in the presence of nigericin. The numbers above each histogram represent buffer pH values. (b) A calibration curve for SNARF-calcein in which the buffer pH was plotted vs. the mean value from each histogram (see Figure 2a). The straight line fitted to the data was calculated by least-squares linear regression analysis. Correlation coefficient ( $r$ )=0.993.



**Figure 3.** SNARF-calcein/AM concentration plotted against the fluorescence intensities at 575 and 625 nm and the fluorescence ratio. For both individual curves logarithmic regression analysis was used, while for the best-fit line of the ratio linear regression analysis was used ( $\Delta$ , F575;  $\bullet$ , F625;  $\blacksquare$ , F625/575).

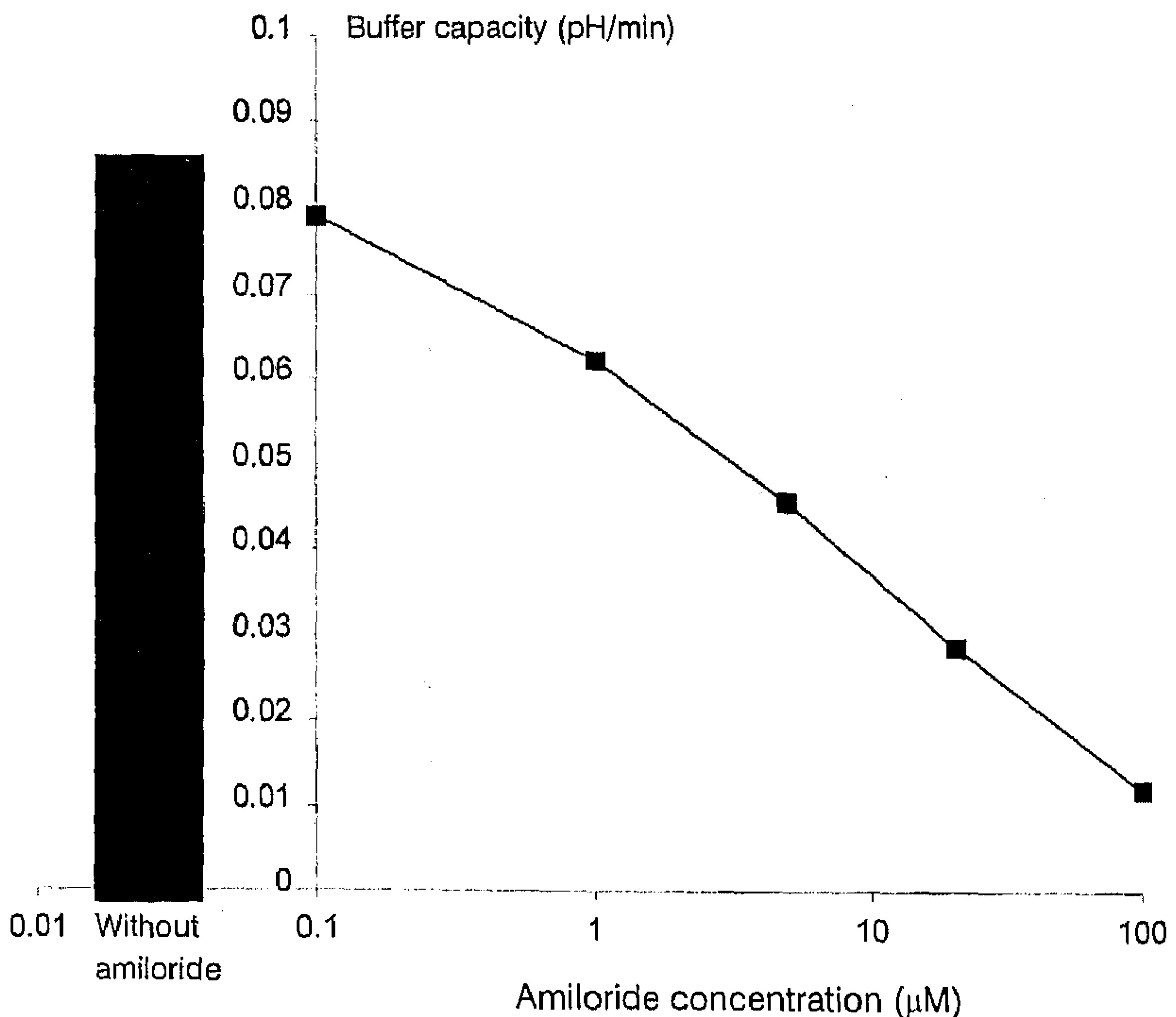


**Figure 4.** Acid load and  $\text{pH}_i$ -recovery experiment. Measurement of physiological pH of SNARF-calcein-loaded cells, addition of  $\text{NH}_4\text{Cl}$  (A), resuspension in  $\text{K}^+$  buffer (B), and finally resuspension in  $\text{Na}^+$  buffer (C). The 1023 channels of the x-axis represent a time period of 30 min. The fluorescence ratio F625/F575 was plotted on the y-axis as a measure for the  $\text{pH}_i$ .

Figure 6 showed DNA histograms and FRHs of proliferating (Figures 6a and c, respectively) and quiescent (Figures 6b and d, respectively) BALB/MK cells. Three days after plating, keratinocytes were switched to either complete or maintenance medium, respectively. After 24 h the cells were harvested at about 85% confluence. A significant difference could be seen in the DNA histograms of proliferating (a) and quiescent (b) cells. The graphs shown are representatives from three individual experiments. The percentage cells in S and G<sub>2</sub>+M phase of the cell cycle was dramatically decreased in the quiescent cell population. The proliferating cell population (c) had a large fraction of cells with relative high pH<sub>i</sub> (pH 7.4), whereas in the quiescent cell population (d) this fraction was almost absent.

To determine whether the state of confluence influences the pH<sub>i</sub> measurements, we compared proliferating and quiescent cultures at low and very high confluences (about 40% and 90%, respectively). The state of confluence appeared to have a strong influence on pH<sub>i</sub> measurements of proliferating cells, but it did not affect pH<sub>i</sub> measurements of quiescent cultures (Figure 7). The pH<sub>i</sub> difference found between proliferating and quiescent cell populations proved to be bigger in cultures with very high confluence compared to cultures with low confluence.

The percentage IdUrd-positive cells was determined as a control for the proliferative activity of the cultured keratinocytes. About 4 h after re-feeding the cultures a pulse label for 30 min with 10 μM IdUrd was given. Cultures re-fed in complete medium showed 53.1% IdUrd-positive cells (*n*=2), while re-fed in maintenance medium showed 15.2% IdUrd-positive cells (*n*=2).

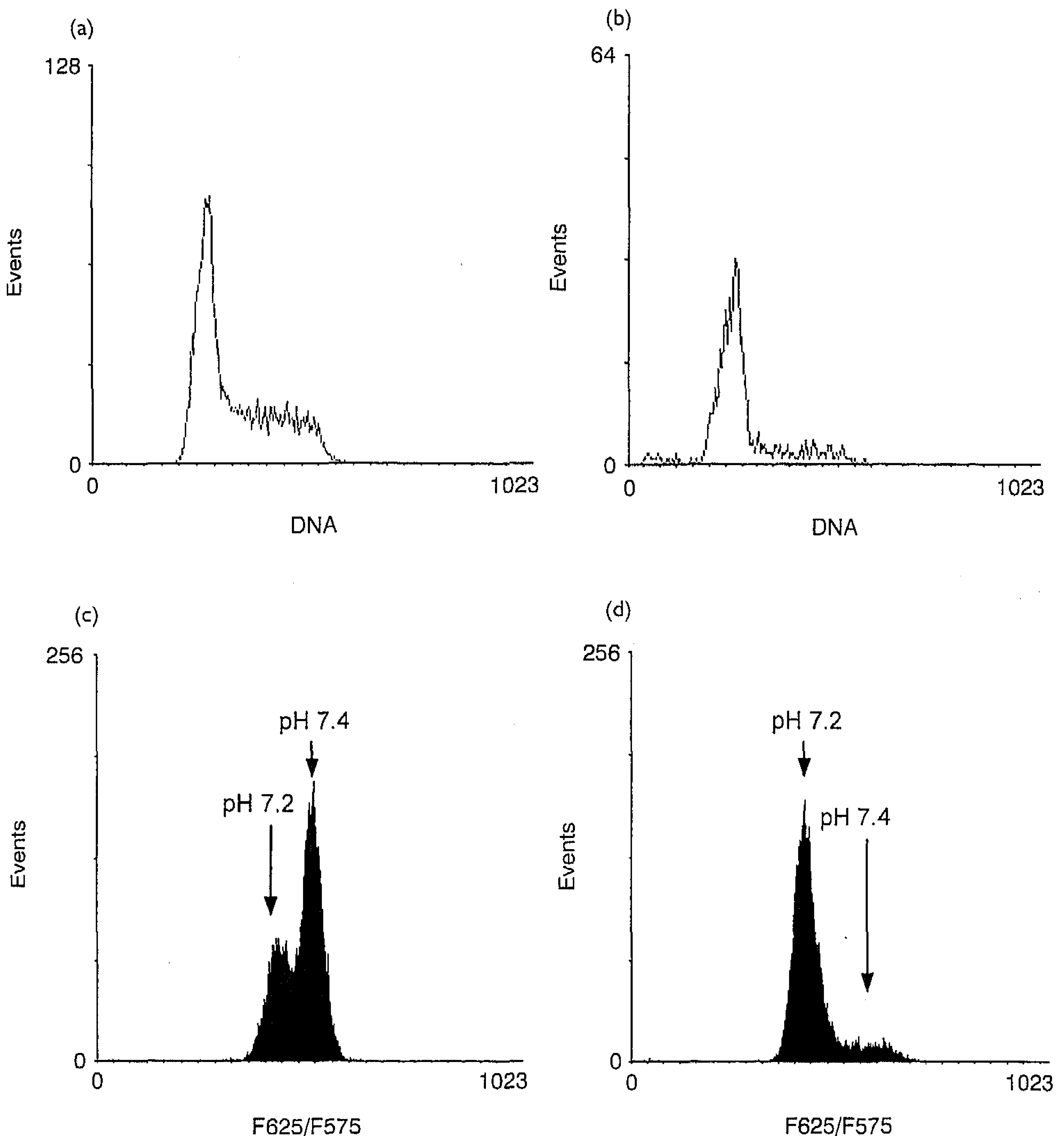


**Figure 5.** Effect of different amiloride concentrations (x-axis) on the pH<sub>i</sub>-recovery process expressed as buffer capacity (pH/min, y-axis). The solid bar represents the buffer capacity of keratinocytes without amiloride.



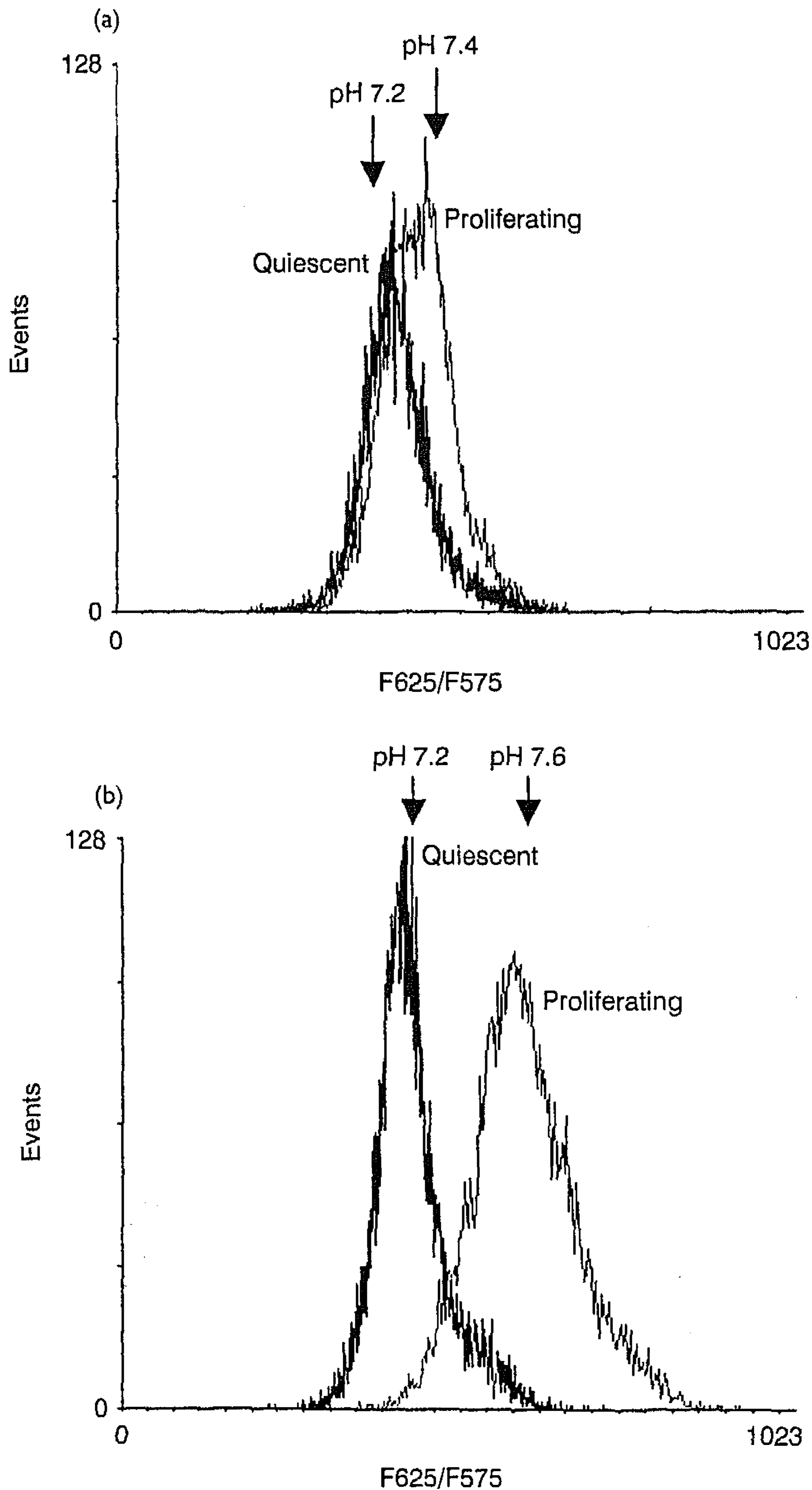
## DISCUSSION

In normal human skin production of keratinocytes and loss by scaling is tightly regulated. The number of proliferating cells in the germinative compartment of the epidermis is relatively low, about 10%. In hyperproliferative skin, e.g. psoriasis, nearly all germinative cells are cycling (Boezeman, Bauer & de Grood 1987, van Erp *et al.* 1996). Whether this is a primary abnormality in this disease and how epidermal proliferation is precisely controlled has poorly been understood. The mouse keratinocyte cell line, BALB/MK, was used to optimize the technique for measuring  $pH_i$  and the association of cytoplasmic alkalization with proliferation was demonstrated.



**Figure 6.** The upper graphs represent DNA histograms of proliferating (a) and quiescent (b) BALB/MK cells. The FRHs of SNARF-calcein-loaded cells under both these conditions are shown in the lower graphs (c and d, respectively). The pH values of the peaks are indicated. These graphs are representatives from three experiments.

One of the first signs, which occurs when cells become activated and start to divide, is a small increase in cytoplasmic pH (Gerson & Kiefer 1983, Pouyssegur *et al.* 1985, Musgrove *et al.* 1987). In a previous study we evaluated the two fluorochromes BCECF and SNARF-1 to determine their capabilities and limitations measuring  $pH_i$  by the fluorescent indicator



**Figure 7.** The data shown are FRHs of SNARF-calcein-loaded proliferating and quiescent keratinocytes at low confluence (a) and very high confluence (b). These graphs are representatives from three individual experiments. In Figure 7a the same biphasic distribution can be observed as shown in Figure 6c.

technique (van Erp *et al.* 1991). Both excitation and emission spectra of BCECF were pH-sensitive. Relative pH-sensitivity was maximal at the peak fluorescence wavelengths (488 nm for excitation and 525 nm for emission) and minimal at the tails of the curves (Grabner *et al.* 1986). A strong increase in emission intensity was seen with increasing pH. Between pH 6 and 8 the spectra of SNARF-1 showed a pH-dependent emission shift from 580 nm to 630 nm and showed an iso-emission point at 610 nm. Because of this spectral shift, it was possible to calculate a ratio of two separate wavelength-bands. In that way the ratio-measurements could be used to relate histogram channel numbers to  $\text{pH}_i$ . An advantage of using a ratio method is that the  $\text{pH}_i$  measurements are independent of differences in cell size, dye uptake, photobleaching, or dye leakage (Thomas *et al.* 1979). This is especially important for heterogeneous cell types, such as keratinocytes. However, the limited linear range of SNARF-1 (between pH 6.8 and 8.0) makes it less suitable for acid-load recovery experiments.

Therefore, we have explored the possibility of using a cocktail of BCECF and SNARF-1. A strong increase in emission intensity at 525 nm for BCECF and at the same time a strong decrease for SNARF-1 at 575 nm with increasing pH, indicated that a cocktail approach could be used to increase the resolution of the method over a broad pH range. Martinez Zaguilan *et al.* successfully used a similar approach with FURA-2, a  $\text{Ca}^{2+}$  indicator, and SNARF-1 as a cocktail (Martinez Zaguilan *et al.* 1991). However, our cocktail approach appeared to be not optimal because of differential uptake and binding kinetics of both dyes. The uptake of BCECF/AM was delayed compared to SNARF-1/AM. In an earlier study we already showed more binding of SNARF-1 to cellular structures (van Erp *et al.* 1991).

Subsequently we tested the novel fluorochrome SNARF-calcein. The spectra for SNARF-calcein excited by 488 nm showed a pH-dependent emission shift from 585 nm at pH 5.5 to 610 nm at pH 8.0 (Figure 1). The fluorescence intensity below 600 nm increased at decreasing pH, whilst the intensity above 600 nm decreased. The FRHs of SNARF-calcein (Figure 2a), compared to SNARF-1, showed a better resolution especially over a larger pH range (pH 6.3–8.0). Calibration curves for SNARF-calcein (Figure 2b) were performed with every  $\text{pH}_i$  experiment to calculate  $\text{pH}_i$  values from the measured fluorescence ratio of a sample. We demonstrated that  $\text{pH}_i$  measurements were independent of dye load. Most essential was that the fluorescence ratio plotted against the SNARF-calcein/AM concentration showed a horizontal line over a concentration range from 1 to 10  $\mu\text{M}$  (Figure 3). This because of the fact that the diameter of keratinocytes can vary between 8 and 25  $\mu\text{m}$  (Gommans *et al.* 1979).

Since SNARF-calcein showed the broadest linear range, this dye was used to demonstrate the existence of a  $\text{NH}_4^+/\text{H}^+$  exchanger in the plasma membrane of BALB/MK cells, using the  $\text{NH}_4^+$ -prepulse technique. First, the pH was measured under physiological conditions, and appeared to be 7.2 in these keratinocytes (Figure 4). Second, by adding  $\text{NH}_4\text{Cl}$ , the  $\text{pH}_i$  increased 0.3 pH units. Because  $\text{NH}_3$  enters the cell more rapidly than  $\text{NH}_4^+$ , the early effects of  $\text{NH}_4^+$  entry could be ignored. The cause of the  $\text{pH}_i$  increase is that  $\text{NH}_3$  enters the cell where it consumes protons in forming  $\text{NH}_4^+$ . A rapid efflux of  $\text{NH}_3$  on changing to  $\text{K}^+$  buffer, without ammonium, then leaves an excess of  $\text{H}^+$  within the cell. Therefore the  $\text{pH}_i$  drops to about 6.0. The  $\text{Na}^+/\text{H}^+$  exchanger, if it exists, could not be activated because  $\text{NH}_4^+$  was replaced by  $\text{K}^+$ . After resuspending the cells in  $\text{Na}^+$  buffer the  $\text{pH}_i$  recovered to its physiological value within about 10 min. This  $\text{Na}^+$ -dependent recovery proved to be amiloride-sensitive (Figure 5). The addition of different concentrations of amiloride before  $\text{Na}^+$  was added, resulted in a dose-dependent inhibition of the recovery. From these observations we deduced that BALB/MK cells possess a  $\text{Na}^+/\text{H}^+$  exchanger in their plasma membrane, which

could contribute to the maintenance of the  $\text{pH}_i$ . Significant differences were found in the DNA histograms and the FRHs of proliferating (Figures 6a and c, respectively) and quiescent (Figures 6b and d, respectively) cells. When there appeared to be many cells in the S and  $G_2+M$  phases a large population of cells with relative high  $\text{pH}_i$  was observed. In contrast, when these cells were absent, the population contained predominantly cells with relative low  $\text{pH}_i$ . This suggests that an increase in  $\text{pH}_i$  is associated with cell proliferation in BALB/MK cells. Such an association has been shown previously (Musgrove *et al.* 1987).

Initial sorting experiments were hampered by the fact that the cultures were harvested at different stages of confluence (data not shown). Furthermore, it is uncertain, whether  $\text{pH}_i$  conditions remain stable over the time period of sorting (2–4 h) (Valet *et al.* 1981). The moment in time to harvest the cultures appeared to be critical. To determine to what extent the stage of confluence affects the  $\text{pH}_i$  measurements, we compared proliferating and quiescent cultures at low confluence (about 40%) and very high confluence (about 90%). These experiments showed that when proliferating cells grow to confluence the  $\text{pH}_i$  increases significantly (Figure 7). In proliferating cultures, some cells still remain quiescent, as can be observed in Figures 6c & 7a. The biphasic distribution of the proliferating population was overlaid by the quiescent population. On the contrary, the  $\text{pH}_i$  of keratinocytes in the quiescent state was independent of the culture conditions used. BALB/MK cells with relative high  $\text{pH}_i$  still proliferate under confluent conditions. Namely, in the mouse keratinocyte cell line the growth fraction remains very high (about 85%) although the cultures reach confluence (van Hooijdonk *et al.* 1993).

In conclusion, culture conditions were critical for studies in which  $\text{pH}_i$  measurements were performed. The novel pH-sensitive dye SNARF-calcein proved to be very suitable for  $\text{pH}_i$  measurements in mouse keratinocytes. Using SNARF-calcein we were able to show proliferation was associated with increased  $\text{pH}_i$ . The  $\text{Na}^+/\text{H}^+$  exchanger could be involved in this process, since acid load and  $\text{pH}_i$ -recovery experiments showed the alkalization to be amiloride-sensitive.

#### ACKNOWLEDGEMENTS

We thank Dr S.A. Aaronson for providing the BALB/MK cell line.

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