Imaging of respiratory-related population activity with single-cell resolution

Frank Funke, Mathias Dutschmann, and Michael Müller

DFG Research Center for Molecular Physiology of the Brain (CMPB), Zentrum für Physiologie und Pathophysiologie, Abteilung Neuro- und Sinnesphysiologie, Georg-August-Universität Göttingen, Göttingen, Germany

Submitted 9 May 2006; accepted in final form 1 September 2006

Funke F, Dutschmann M, Müller M. Imaging of respiratory-related population activity with single-cell resolution. Am J Physiol Cell Physiol 292: C508–C516, 2007.—The pre-Bötzinger complex (PBC) in the rostral ventrolateral medulla contains a kernel involved in respiratory rhythm generation. So far, its respiratory activity has been analyzed predominantly by electrophysiological approaches. Recent advances in fluorescence imaging now allow for the visualization of neuronal population activity in rhythmogenic networks. In the respiratory network, voltage-sensitive dyes have been used mainly, so far, but their low sensitivity prevents an analysis of activity patterns of single neurons during rhythmogenesis. We now have succeeded in using more sensitive Ca2+ imaging to study respiratory neurons in rhythmically active brain stem slices of neonatal rats. For the visualization of neuronal activity, fluo-3 was suited best in terms of neuronal specificity, minimized background fluorescence, and response magnitude. The tissue penetration of fluo-3 was improved by hyperosmolar treatment (100 mM mannitol) during dye loading. Rhythmic population activity was imaged with single-cell resolution using a sensitive charge-coupled device camera and a ×20 objective, and it was correlated with extracellularly recorded mass activity of the contralateral PBC. Correlated optical neuronal activity was obvious online in 29% of slices. Rhythmic neurons located deeper became detectable during offline image processing. Based on their activity patterns, 74% of rhythmic neurons were classified as inspiratory and 26% as expiratory neurons. Our approach is well suited to visualize and correlate the activity of several single cells with respiratory network activity. We demonstrate that neuronal synchronization and possibly even network configurations can be analyzed in a noninvasive approach with single-cell resolution and at frame rates currently not reached by most scanning-based imaging techniques.

A RHYTHMOGENIC NEURONAL NETWORK with intrinsic voltage-dependent bursting properties is located in the pre-Bötzinger complex (PBC) of the rostral ventrolateral medulla (1, 16, 17, 30, 35). The respiratory rhythms of the in vitro PBC were previously studied in transverse slice preparations of 250- to 700-μm thickness (30, 32, 35), tilted-sagittal slice preparations (28), as well as in en bloc preparations (22, 37). The location of the primary respiratory rhythm generator and a potential role for pacemaker neurons in the generation of breathing rhythms remain controversial though (13). A recent study provides evidence that pacemaker activity is exclusively associated with gasping patterns in situ (27). The current hypotheses regarding respiratory rhythm generation are largely based on electrophysiological studies, which yield excellent temporal resolution of single-cell activities, but analysis of population activity is difficult.

With the recent advances in imaging techniques, the PBC, as well as other respiratory centers, have been studied optically using voltage-sensitive dyes in isolated brain stem spinal cord preparations (8, 23, 25, 39) and even in the perfused brainstem preparation (9, 29). However, respiratory network functions and the activity of several network elements could not be probed at single-cell resolution. To image population activity in a tissue slice with sufficient spatial and temporal resolution, it is crucial to ensure that changes in fluorescence intensity can be observed without excessive averaging. It is also important that the tissue penetration of the dye is sufficient to reach deeper cell layers and to label a maximum number of cells. At the same time, background fluorescence and staining of cells other than neurons should be negligible. Voltage-sensitive dyes (such as di-2-ANEPQ or di-4-ANEPPS) are of moderate molecular size (549 and 481 g/mol, respectively), ensuring sufficient tissue penetration, but their response magnitudes to membrane potential changes are rather small (∼10% intensity change/100 mV). Therefore, a reasonable signal-to-noise ratio can only be obtained by averaging their responses over several activity cycles. Nevertheless, with such averaging approaches, respiratory centers and the propagation of neuronal activity could be reliably visualized in the en bloc and the perfused preparation (9, 24, 25, 29), yet without obtaining single-cell resolution.

In the present study we used Ca2+-sensitive dyes to study in vitro rhythmogenesis. We have chosen this type of indicator because the intracellular Ca2+ concentration ([Ca2+]i) reliably reflects single-cell activity (31). Ca2+-sensitive dyes exhibit a larger molecular size than voltage-sensitive dyes (>1000 g/mol) but they yield much higher response magnitudes, with the activity-related rises in [Ca2+]i, yielding often more than a 10-fold increase in fluorescence emission (20). These dyes already have been used in previous studies on rhythmic PBC slices (11, 15, 21), in combination with confocal and multiphoton imaging, as well as wide-field microscopy (19, 32). However, loading of the neurons with Ca2+ indicators seemed to be a limiting factor. The dyes had to be injected into the tissue (16), or very high dye concentrations were used to incubate slices (19).

We imaged respiratory-related population activity with single-cell resolution, using fluo-3 AM and charge-coupled device (CCD) camera imaging. Fluorescence detection by CCD camera guarantees maximum flexibility in the choice of optical components and it maintains the advantage of frame rates of 20 Hz or higher. It also provides the excellent quantum efficiency...
of CCD chips and allows for fast wavelength switching in the millisecond range, an absolute requirement for ratiometric approaches and multiparametric recordings. Here we present the first promising imaging results and give details on the offline image processing procedures that helped to improve detection of single neurons within rhythmically active slice preparations. Correlation of optically detected cellular activity with extracellularly recorded PBC mass activity (field potential) allows for the unequivocal classification of neuronal subtypes as well as the analysis of their synchrony and modulation during in vitro rhythmogenesis. Two neuronal subtypes could be distinguished optically: inspiratory neurons showing a $[\text{Ca}^{2+}]_i$ rise in coincidence with PBC mass activity, and expiratory neurons showing minimum $\text{Ca}^{2+}$ fluorescence during the PBC burst, but producing a $[\text{Ca}^{2+}]_i$ rise during the interburst interval.

**MATERIALS AND METHODS**

**Preparation.** Rhythmically active slices were prepared from juvenile Sprague-Dawley rats (postnatal day 2–8, either sex). Rats were deeply anesthetized with isoflurane and decapitated, and the brain was rapidly removed and transferred to chilled (5°C) artificial cerebrospinal fluid (ACSF). The pia mater was removed, the isolated brainstem was glued on an agarose block, and a transverse 700-μm slice containing the PBC was cut with a vibroslicer (752M; Campden Instruments) at an angle of 25°. The slice was transferred to ACSF (30°C) and was left undisturbed for at least 1 h before dye loading.

**Solutions and fluorescent indicators.** Unless mentioned otherwise, all chemicals were obtained from Sigma Aldrich. ACSF was composed of (in mM) 128 NaCl, 3 KCl, 1.5 CaCl$_2$, 1 MgSO$_4$, 0.5 NaH$_2$PO$_4$, 21 NaHCO$_3$, and 30 dextrose. It was constantly aerated with carbogen (95% O$_2$–5% CO$_2$) to adjust pH to 7.4. Rhodamin123 (Rh123) was dissolved in ethanol as a 20 mg/ml stock. Calcium green-2 AM (Molecular Probes), fura-2 AM (Molecular Probes), Oregon green BAPTA-1 AM (Molecular Probes), and fluo-3 AM (Mobitec) were dissolved in DMSO containing 10% pluronic acid as standard deviation (SD), and the number of observations ($n$) is given. Statistical calculations were done with Excel software (Microsoft Office 2003).

**RESULTS**

Neuronal activity is associated with characteristic changes in metabolic markers (NADH/FAD), mitochondrial membrane potential, cytosolic ion levels, membrane potential, and cell volume, all of which can be monitored optically. We considered cytosolic Ca$^{2+}$ levels to be the best optical marker for neuronal activity, since [Ca$^{2+}$], changes have been shown in various preparations to truthfully reflect activity changes, especially in respiratory neurons in which spike discharges are grouped into long-lasting bursts (11, 15).

The penetration of a fluorescent dye into the tissue is determined by its molecular size and structure (its hydrodynamic profile to be more precise), tortuosity of the interstitial space, lipid solubility, activity of intracellular esterases, extrusion by cellular anion transport systems, as well as the tendency of the single dye molecules to form aggregates. Therefore, the suitability of a given dye is difficult to predict and rather has to be evaluated empirically. In initial trials we therefore tested various Ca$^{2+}$ indicators to identify the fluorescent dye best suited for the optical detection of neuronal activity within the PBC. Since the [Ca$^{2+}$], changes of respiratory neurons have been shown previously to reach peak levels of 300 nM (11), we concentrated on those dyes that exhibit low
binding constants ($K_d = 145–550$ nM) and reliably respond to even small changes in $[\text{Ca}^{2+}]_i$. Also, we have chosen dyes that are characterized by longer excitation and emission wavelengths. At longer wavelengths, light scattering within the tissue is less pronounced, which might be favorable for the photon detection efficiency of the optical approach. Bulk loading of slices with Calcium green-2 ($n = 4$) or Oregon green BAPTA-1 ($n = 4$) resulted in a high background fluorescence, and predominantly glial cells appeared to be labeled. With fura-2 loading ($n = 5$), the background fluorescence was somewhat lower and a more favorable neuron/glia ratio was obtained, but with none of these dyes did we observe optically detected neuronal activity that clearly correlated with PBC mass activity. The mitochondrial membrane potential marker Rh123 (5, 7) was tested as well. Rh123 is a membrane-permeable cationic dye that accumulates in mitochondria (7), because these are usually the most negative sites within the cell. Changes in mitochondrial membrane potential cause a
redistribution of Rh123 across the inner mitochondrial membrane, thereby giving rise to changes in Rh123 fluorescence. Pronounced cell activity, such as bursting behavior, massive excitation, or metabolic insults all do affect Rh123 fluorescence (10), and moderate changes in Rh123 fluorescence during respiratory activity were reported as well (21). Based on these results and also due to its small molecular size, we tested whether Rh123 is suitable as an extracellularly applied activity marker in acute slices. However, the intense Rh123 labeling and the high background fluorescence of the superficial cell layers made it difficult to detect reliable optical signals from the tissue. Of all tested dyes, only fluo-3 labeled mostly neurons, and their rhythmic activity clearly correlated with PBC mass activity (Fig. 1). Therefore, we considered fluo-3 as the ideal activity marker and used it for further experiments.

Dye penetration depth and cell labeling. Rhythmically active neurons were easily detectable in the slices (Fig. 1). Due to slow dye diffusion into the tissue, superficial cells were more intensely labeled compared with those in deeper tissue layers (Figs. 1A and 2B). Crucial for our imaging approach was to estimate the tissue penetration of fluo-3 and the quantity of labeled cells. Focusing into the tissue in 2-μm steps revealed that fluo-3 (3 μM, 45 min at 30°C) labels cells only to a depth of 39 μm (SD 6.5, n = 5, Fig. 2A). In an attempt to improve dye penetration into deeper tissue layers, we increased the dye concentration up to 10 μM or prolonged the dye loading time to 1 h. Alternatively, we varied the loading temperature (4–30°C) to modify the activity of intracellular esterases that revert the dye into the fluorescent product once it enters the cell. However, none of these attempts showed any obvious improvement of labeling. Probenecid, an inhibitor of anion transport and MDR (multidrug resistance transporter) was reported to prevent dye extrusion by cellular transport systems and to improve labeling of olfactory receptor neurons of Xenopus laevis tadpoles (18). Yet in our rhythmically active medullary slices, fluo-3 loading was not noticeably improved by probenecid (2.5 mM). Since the above negative results suggest that the tortuosity of the interstitial space is the limiting factor for the tissue penetration of fluo-3, we increased the osmolarity during dye loading to reduce cellular volume and thereby widen the interstitial space. Testing different degrees of hypertonicity revealed that fluo-3 loading improved significantly when 100 mM mannitol were added, and that additional cells in deeper tissue layers were labeled. Following mannitol treatment, labeled cells were found at a depth of 56.9 μm (SD 4.6, n = 8, Fig. 2A). Higher concentrations of mannitol (>100 mM) reduced, however, the viability of slices. It should be noted that mannitol was present during dye loading only. The experiments themselves were performed with standard ACSF of normal osmolarity.

To compare the amount of fluo-3-labeled cells with the total number of cells present, we performed double labeling with the nuclear stain DAPI (3 μM, 30 min. at 30°C), which labels every cell. Detailed analysis showed that fluo-3 labeled ~30% of the cells within the upper 60 μm of the slice (Fig. 2B). Due to the better tissue penetration of the nuclear stain, DAPI-labeled cells were detectable down to a depth of 141.7 μm (SD 2.9, n = 3, Fig. 2A). This clearly shows that the current detection depth of the imaging approach is not determined by absorption or scattering of the emitted photons, but rather by the penetration depth of the fluorescent dye.

![Fig. 2. Tissue penetration of fluorescent markers and efficiency of staining. A: fluo-3 labeling was restricted to the upper layers of the slices. Focusing into the tissue revealed that fluo-3-labeled cells within the upper 40 μm of the slice (without mannitol). Labeling performed under hyposomotic conditions, induced by adding different concentrations of mannitol, improved the penetration depth of fluo-3 to ~65 μm. Best results were obtained with 100 mM mannitol. In contrast, the nuclear marker 4,6-diamidino-2-phenylindole (DAPI) penetrated the tissue more easily, being detectable up to a depth of 140 μm. The displayed images are xz-plane projections of the single images taken at a plane distance of 2 μm. B: PBC region of an acute slice labeled with the Ca2+ indicator fluo-3. Bulk loading of slices labeled only a certain amount of total cells present. DAPI counterstaining was used to determine the total number of cells within the field of view, and it shows that ~30% of cells were labeled by fluo-3.](http://ajpcell.physiology.org/ by 10.220.33.4 on May 30, 2017)
**Imaging respiratory-related activity.** Even though the PBC can be isolated in the rhythmically active slice, this represents a reduced preparation containing only parts of the respiratory control centers being deprived of most of their excitatory afferents. Therefore, raising extracellular K⁺ concentration ([K⁺]₀) and thereby increasing neuronal excitability is required to elicit and stabilize the spontaneous respiratory activity (34, 35). Rhythmically active slice preparations under such high [K⁺]₀ exhibit a largely reduced activity pattern compared with the intact network. Therefore, the inspiratory and expiratory neuronal activity recorded in the present study may not necessarily reflect a normal in vivo breathing pattern. Recently the group of Ballanyi succeeded in obtaining respiratory-active slices of reduced thickness (down to 250 μm) at normal, 3 mM [K⁺]₀, at least for a limited duration of time (32). Nevertheless, to ensure reliable recording conditions, we progressively elevated [K⁺]₀ from 3 to 8 mM and obtained a stable PBC rhythm that was recorded for 3–6 h. The average respiratory burst frequency was 7.6 bursts per min (SD 2.2, n = 24 slices). Up to nine rhythmically active neurons, usually three neurons, were detected in the field of view (≈450 × 340 μm, ×20 objective). Since fluo-3 has a very large response magnitude, it visualizes reliably the activity pattern of individual neurons in relation to a single respiratory burst. Respiratory-related optical neuronal activity was obvious online in 29% of slices, and other rhythmically active neurons became detectable after

---

**Fig. 3.** Optically detected inspiratory and expiratory activity. A: the sequence of images shows the optical activity of three inspiratory and one expiratory neuron. The displayed images were taken 0.5 s before until 2 s after the detection of the PBC burst, and they were averaged over 32 consecutive bursts. Due to offline processing, an increase in [Ca²⁺]ᵢ above the average concentration is indicated by bright pixels, while darkening of the pixels indicates decreased [Ca²⁺]ᵢ levels (see MATERIALS AND METHODS for details). Red, green and orange arrowheads mark inspiratory neurons while the blue arrowhead marks an expiratory neuron. Note that for the red-marked neuron, cellular processes are visible in frames 3 and 4. B: exact time point of the individual images (see A, frames 1–6) in the Ca²⁺ recording and its relation to the PBC burst. C: optically recorded changes in [Ca²⁺]ᵢ (fluor-3 emission) of an expiratory and an inspiratory neuron in correlation with PBC mass activity. The traces show the oscillatory behavior of the expiratory and the inspiratory neurons. The more intense fluor-3 signal of the inspiratory neuron is due to its more superficial location. Note that in the expiratory neuron, [Ca²⁺]ᵢ was elevated during the entire interburst interval, while it decreased during the PBC burst. In contrast, in the inspiratory neuron, [Ca²⁺]ᵢ was low during the interburst and increased during the burst.
offline image processing. Correlation of the optical activity pattern with the simultaneously recorded PBC mass activity (recorded in the contralateral PBC to prevent damage of the imaged area) allowed for the identification of different cell types (Figs. 3 and 5). The majority of cells were classified as inspiratory neurons (74%, n = 55 cells), with their most intense Ca$^{2+}$ fluorescence coinciding with PBC mass activity. In inspiratory neurons (26%, n = 19 cells), the minimum Ca$^{2+}$ fluorescence coincided with PBC mass activity, while [Ca$^{2+}$], was elevated during the interburst interval (Figs. 3 and 5A). The inspiratory and expiratory neurons were neither grouped nor clustered, but they appeared rather randomly distributed within the field of view. An example of respiratory single-cell activity is illustrated in Fig. 3. The images displayed in Fig. 3A show four rhythmically active cells, three inspiratory neurons (red, green, and orange arrowheads), and one expiratory neuron (blue arrowhead). One inspiratory neuron (red) and the expiratory neuron (blue) were visible online during the PBC burst, and even axonal/dendritic structures of the inspiratory neuron were detectable. (Fig. 3A). The green- and the orange-marked, inspiratory neurons are located in deeper tissue layers and became detectable only upon offline image processing (Fig. 3B). Note that all neurons displayed stable oscillatory behavior (Fig. 3C, also Fig. 5A).

To exclude the possibility that optical signals from glial cells contribute to the recorded respiratory-related activity we identified glial cells by lowering [K$^+$]o, to 0.2 mM, a treatment that has been reported to induce glial Ca$^{2+}$ oscillations (4). As expected, the neuronal respiratory-related Ca$^{2+}$ oscillations were abolished, while putative glial cells responded with long-lasting Ca$^{2+}$ transients (Fig. 4, n = 4). The glial cells identified by such treatment never showed any rhythmic activity that clearly correlated with PBC mass activity. In contrast, neurons that were found to be active in correlation with PBC mass activity never responded with massive Ca$^{2+}$ transients in response to low [K$^+$]o. In the optically identified glial cells, we occasionally observed increases in [Ca$^{2+}$], which were, however, neither periodic nor phase locked to the PBC rhythm. Visualizing in vitro network interactions. Visualizing the activity of several rhythmically active neurons should allow for the study of network interactions during in vitro rhythmogenesis. This is verified by proof-of-principle measurements. Interestingly, we observed that a spontaneous increase of [Ca$^{2+}$], in inspiratory neurons resulted in a transient suppression of [Ca$^{2+}$], oscillations in a simultaneously recorded inspiratory neuron and most importantly caused a significant prolongation of the PBC burst interval (Fig. 5A). This recording suggests that certain cell types of the in vitro network are potentially able to trigger a spontaneous phase reset. This underlines the potency of our imaging approach, since the activity pattern of several cells could be investigated. Such spontaneous phase resetting triggered by expiratory neurons of the PBC was potentially overlooked in previous studies, although fluctuations in the burst interval were obvious. Like in previous studies (17), we observed spontaneously augmented PBC burst activities. Analysis of the underlying cellular activity revealed that this augmentation involves a so far not described recruitment of neurons that solely followed the rhythm of the augmented PBC bursts (Fig. 5B).

In addition, our imaging approach confirms interesting phenomena on the cellular level. The obtained recordings demonstrate that inspiratory neurons regularly show double bursting that is not detectable in the PBC mass activity (arrowheads in Fig. 5B). In summary, our imaging approach using fluo-3 in combination with CCD camera imaging is able to detect population activities with single-cell resolution that could not be revealed by conventional single-cell, patch-clamp recordings, or with imaging techniques requiring excessive offline averaging.

**DISCUSSION**

Using CCD camera imaging and Ca$^{2+}$-sensitive dyes we were able to visualize the activity pattern of putative respiratory neurons in vitro. The novelty of our approach is that we are able to identify several cells and analyze their activity pattern at single-cell resolution. This allows for...
spatiotemporal studies of neuronal activity and synchrony in spontaneously active neural networks using a noninvasive approach.

Of the various Ca\(^{2+}\)-sensitive dyes tested, we found fluo-3 to be the best suited marker for the visualization of active respiratory neurons in acute brain stem slices. With the low concentration used, a high specificity of neuronal vs. glial labeling was obtained, and structures down to \(\sim 60\ \mu\text{m}\) in the tissue could be labeled. Also, the risk of unintentionally affecting the intracellular calcium buffering by the presence of the dye was minimized. Nevertheless, the dye-loading strategy still requires further improvement to optimize the tissue penetration of the dye. As performed in other studies (16, 36), the dye could be injected into the tissue close to the recording site. However, placement of the injection pipette might cause mechanical damage and disturb the subtle interactions of the isolated network. Alternatively, novel Ca\(^{2+}\) indicators with improved response magnitudes, such as fluo-4 NW, might increase the sensitivity of our imaging approach. In terms of tissue penetration and its determining factors discussed earlier, the availability of Ca\(^{2+}\) indicators of smaller molecular size would be advantageous, as demonstrated, for instance, by the superior tissue penetration of the much smaller nuclear marker DAPI (Fig. 2).

Earlier imaging studies already came close or even reached single-cell resolution; however, in these studies the dye was

---

**Fig. 5. Visualization of network interactions.**

A: correlation of neuronal population activity with optical recordings reveals real-time network interactions with single-cell resolution. The fluo-3 traces (raw signals) show the activity of an expiratory and an inspiratory neuron with oscillatory activity patterns. A spontaneous, pronounced increase in \([\text{Ca}^{2+}]_i\), of the expiratory neuron (arrow) coincides with a depression of inspiratory neuronal activity accompanied by a prolonged interburst interval. Note that the size of the following PBC burst is reduced as well. This recording suggests that expiratory neurons of the in vitro network are potentially able to trigger a phase reset. B: optical recording of apparently functionally different cell types. Augmented PBC bursts are generated by neuronal recruitment. The upper trace shows the \([\text{Ca}^{2+}]_i\) oscillations of a neuron that is only active during augmented PBC bursts, while the inspiratory neuron shown in the middle trace basically followed each PBC burst. However, this neuron also displayed some bursting activity not correlated with PBC bursting (arrowheads). This underlines the capability of our imaging approach to precisely identify cellular activity patterns in correlation with in vitro network activity.
either directly loaded into only one cell (11) or it was injected into the midline of the slice, followed by retrograde labeling of respiratory neurons within 8–12 h (16). The latter approach has the advantage that neurons in deeper layers also obviously became labeled; however, the long delay required for dye diffusion back into the soma might reduce the viability of the tissue under investigation.

Wide-field microscopy vs. confocal laser scanning microscopy. The use of a standard fluorescence (xenon bulb) light source and CCD camera imaging has certain advantages over laser illumination and scanning-based techniques. CCD cameras have excellent sensitivity (quantum efficiency 0.7), and they are capable of high frame rates (~10 Hz for full frames, up to ~100 Hz with binning and selected regions of interest). The xenon light source provides the entire visible wavelength spectrum, including ultraviolet and near-infrared components (at least 300–800 nm); therefore, virtually any dye can be used. Most importantly, however, this flexibility of wavelength selection comes with the opportunity for fast wavelength switching (within less than 2 ms), which offers the possibility of ratiometric approaches and the (almost) simultaneous detection of multiple optical parameters from a given sample (see, e.g., DAPI and fluo-3 emission in Fig. 2B). Also, as excitation and emission are spectrally close in one-photon excitation, fewer restrictions apply to the objectives. Even a combination with structured illumination would be possible, thereby yielding an axial resolution that is almost comparable to confocal approaches (12).

Perspectives of the method. Visualizing the neuronal activity pattern within the spontaneously active kernel of the respiratory network allows for the noninvasive, simultaneous analysis of several neurons and glial cells, an advantage that can hardly be achieved by classical electrophysiological approaches. Sharp electrode or whole-cell recordings are usually limited to single cells only, cause microdamage to the tissue, and affect the cytosolic composition of the cell under investigation.

Analyzing the activity pattern of optically detected neurons revealed interesting insights into in vitro rhythmogenesis. Of the investigated neurons, 26% displayed expiratory activity patterns correlated to inspiratory bursts of the PBC mass activity. This result was surprising, since expiratory neurons have only rarely been reported for slice preparations (17, 38). To some extent this might be due to the 700-μm-thick slice preparation that certainly contains more parts of the medullary ventral respiratory group than thinner slices. Whether the imaged inspiratory neuronal activity arises from the rostral parafacial region, as the proposed generator for expiratory activity (13, 14), is one interesting question for future imaging investigations. Furthermore, our data suggest that the cellular activity in the PBC in vitro is apparently not restricted to inspiratory pacemaker neurons. Also the observed changes in network behavior so far, including spontaneous phase resetting or recruitment of different cell populations for the generation of augmented PBC bursts in vitro, are promising. A particularly interesting perspective is provided by the time resolution of our imaging approach. It allows for a direct comparison of the physiologic properties of the inspiratory and expiratory neurons (e.g., responses to external and internal modulators, metabolic compromise, and electrical stimulation) in a noninvasive manner. Cytosolic composition and signaling is not affected, which is a clear advantage over whole-cell or sharp electrode recordings.

Concluding remarks. In conclusion our optical approach allows for the noninvasive recording of respiratory-related population activity with single-cell resolution, and down to a depth of 60 μm in the isolated PBC network. It offers the opportunity of ratiometric approaches and is feasible for multiparametric recordings to characterize cellular interactions or biophysical properties of glial cells and respiratory neurons in vitro. We hope to adapt Ca2+ imaging of population activity to more complex preparations like the en bloc brain stem spinal cord neurons preparation (37) or the working heart brainstem preparation (26). Both preparations have already been used in imaging approaches by others (9, 24, 29), but due to the use of voltage-sensitive dyes the crucial single-cell resolution could not be obtained. Our long-term goal is to adapt our imaging method to the lateral approach in the neonatal rat (6), to study the contribution, interaction, and hierarchy of the different respiratory centers during eupnea-like network activity in situ.

Hence, single-cell resolution of modern imaging approaches is required to prove or disprove current theories of rhythm generation and pattern formation based on modeling approaches (2, 3, 33, 34). While our focus of interest is on the respiratory network, the technical approach is by no means limited to that brain region, but it may as well prove substantial in the analysis of functional networks in other brain regions or tissues.

ACKNOWLEDGMENTS

We thank Belinda Hildebrandt for expert technical assistance.

GRANTS

This study was supported by grants of the Deutsche Forschungsgemeinschaft (DFG) to M. Dutschmann and M. Müller and by Göttingen University (Ausstattungmittel Juniorprofessur M. Müller, Gerätebeschaffung: CCD imaging system, M. Dutschmann).

REFERENCES

10. Foster KA, Galeffi F, Gerich FJ, Turner DA, Müller M. Optical and pharmacological tools to investigate the role of mitochondria during...