Two-photon microscopy and fluorescence lifetime imaging reveal stimulus-induced intracellular Na\(^+\) and Cl\(^-\) changes in cockroach salivary acinar cells

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Lahn M, Dosche C, Hille C. Two-photon microscopy and fluorescence lifetime imaging reveal stimulus-induced intracellular Na\(^+\) and Cl\(^-\) changes in cockroach salivary acinar cells. Am J Physiol Cell Physiol 300: C1323–C1336, 2011. First published February 23, 2011; doi:10.1152/ajpcell.00320.2010.—The intracellular ion homeostasis in cockroach salivary acinar cells during salivation is not satisfactorily understood. This is mainly due to technical problems regarding strong tissue autofluorescence and ineffective ion concentration quantification. For minimizing these problems, we describe the successful application of two-photon (2P) microscopy partly in combination with fluorescence lifetime imaging microscopy (FLIM) to record intracellular Na\(^+\) and Cl\(^-\) concentrations ([Na\(^+\)], [Cl\(^-\)]) in cockroach salivary acinar cells. Quantitative 2P-FLIM Cl\(^-\) measurements with the dye N-(ethoxycarbonylmethyl)-6-methoxy-quinolinium bromide indicate that the resting [Cl\(^-\)] is 1.6 times above the Cl\(^-\) electrochemical equilibrium but is not influenced by pharmacological inhibition of the Na\(^+-\)K\(^+-\)2Cl\(^-\) cotransporter (NKCC) and anion exchanger using bumetanide and 4,4'-disothiocyanatoethylhydrostilbene-2,2'-disulfonic acid disodium salt. In contrast, rapid Cl\(^-\) reuptake after extracellular Cl\(^-\) removal is almost totally NKCC mediated both in the absence and presence of dopamine. However, in physiological saline [Cl\(^-\)] does not change during dopamine stimulation although dopamine stimulates fluid secretion in these glands. On the other hand, dopamine causes a decrease in the sodium-binding benzofuran isophthalate tetra-ammonium salt (SBFI) fluorescence and an increase in the Sodium Green fluorescence after 2P excitation. This opposite behavior of both dyes suggests a dopamine-induced [Na\(^+\)] rise in the acinar cells, which is supported by the determined 2P-action cross sections of SBFI. The [Na\(^+\)] rise is Cl\(^-\) dependent and inhibited by bumetanide. The Ca\(^{2+}\)-ionophore ionomycin also causes a bumetanide-sensitive [Na\(^+\)] rise. We propose that a Ca\(^{2+}\)-mediated NKCC activity in acinar peripheral cells attributable to dopamine stimulation serves for basolateral Na\(^+\) uptake during saliva secretion and that the concomitantly transported Cl\(^-\) is recycled back to the bath.

epithelial ion transport; Na\(^+\)-K\(^+-\)2Cl\(^-\) cotransporter; MQAE; SBFI; 2P cross section

THE SIGNIFICANCE OF SALIVA SECRETION can be clearly seen in individuals with salivary gland dysfunction such as the autoimmune disease Sjögren’s syndrome or the systemic disease cystic fibrosis (39, 58). However, adequate clinical therapies require a detailed understanding of the complex physiology of the secretion processes in human salivary glands. Animal models such as mouse and rat salivary glands can provide valuable systems for analyzing this aspect (57). In addition, insect salivary glands are also useful model systems because they display benefits over higher mammals regarding rearing, methodological accessibility, and ease of physiological manipulation. The tubular salivary glands of the blowfly Calliphora vicina are an outstanding example for studying the ubiquitous inositol-1,4,5-trisphosphate/Ca\(^{2+}\) signaling pathway and the regulation of the vacuolar-type H\(^+\)-ATPase (5, 10). In the salivary glands of the cockroach Periplaneta americana (P. americana) the excitation-secretion coupling for epithelial transport and the aminergic signal transduction have been investigated comprehensively (31, 77). Similar to mammalian salivary glands, those of P. americana are of the acinar type with a branched network of grape-like acini and an extensive duct system downstream of the acini (34). Salivation is controlled by dopaminergic, serotonergic, and GABAergic neurons (4, 65), and saliva secretion can be stimulated by the biogenic amines dopamine and serotonin (36). In agreement with the two-stage hypothesis of salivation in mammalian salivary glands (9), those in cockroach secrete an isosmotic NaCl-rich primary saliva, which is subsequently modified in the ducts (24, 60). At this, acinar peripheral cells are specialized for electrolyte and water transport, whereas acinar central cells are responsible for secretion of proteins (36, 61).

Although we have knowledge about the neuronal control of salivation and the stimulus-induced saliva composition in the cockroach salivary glands, our understanding of the ion transport systems and signaling cascades involved in salivation especially for the acinar cells is still limited. In diverse insect epithelia such as Malpighian tubules, midgut, and tubular salivary glands fluid secretion is energized by a vacuolar-type H\(^+\)-ATPase located at the apical plasma membrane (6). However, no vacuolar-type H\(^+\)-ATPase has been found in cockroach salivary acinar cells. The primary saliva secretion resulting from transepithelial ion and water transport through the peripheral cells seems to be energized by a Na\(^+\)-K\(^+-\)2Cl\(^-\) cotransporter (NKCC), Cl\(^-\)-HCO\(_3\)\(^-\) (AE), and Na\(^+\)-H\(^+\) exchangers (NHE) within the cockroach salivary acinar peripheral cells is still postulated only indirectly from microfluorometric ion measurements in duct cells and recordings of saliva secretion rates (28, 60). The lack of data concerning acinar ion homeostasis exists mainly because of methodological problems. The cockroach salivary acini are
compact structures with two secretory cell types, the peripheral cells and the central cells (34). This fact makes it difficult to ascribe a recorded cell response to a particular cell type when using ion-sensitive microelectrodes. On the other hand, because of their dense appearance, acini are difficult to load with fluorescent dyes, resulting in a labeling restricted to cells near the acinar surface. Moreover, the acini exhibit a strong auto-fluorescence in the UV and blue spectral range of light, which in addition changes upon dopamine stimulation (59). It has been also shown that stimulation of acinar cells can be accompanied by transient changes in the cell volume (56). Both events manipulate per se the detected fluorescence intensity of ion-sensitive fluorescent dyes independent of changes in the respective ion concentration quantification procedure.

To overcome the methodological problems mentioned above, we performed two-photon (2P) microscopy and fluorescence lifetime imaging microscopy (FLIM). In fact, both innovative techniques have become leading imaging tools in fundamental and applied physiological research, and we have successfully applied these techniques in cockroach salivary glands previously (29, 30). The fundamental advantage of 2P microscopy over conventional wide field or confocal one-photon microscopy is the use of pulsed low-energy, near-infrared laser light for dye excitation. The nonlinear excitation process results in a highly localized fluorescence excitation restricted to the focal volume, which minimizes the generation of autofluorescence and reduces the global photobleaching of the fluorescent dye as well as tissue damage (27). In addition to the fluorescence intensity as common recording parameter when using fluorescence microscopy, the FLIM technique provides access to the fluorescence decay time of a dye. This nanosecond lifetime is mostly independent of the dye concentration, which is influenced by dye loading efficiency, cell volume changes, or photobleaching. Thus FLIM allows quantitative ion concentration measurements of high reliability (72).

By applying 2P microscopy and 2P-FLIM in this study we showed for the first time recordings of intracellular $\text{Na}^+$ and $\text{Cl}^-$ concentrations ([Na$^+$], and [Cl$^-$]) from living acinar peripheral cells of isolated cockroach salivary glands.

MATERIALS AND METHODS

Animals and gland preparation. A colony of the American cockroach $\textit{P. americana}$ (L.) was reared at 27°C under a light/dark cycle of 12 h:12 h at the Department of Animal Physiology. The animals had free access to food and water. Only male adults aged between 4 and 6 wk were used for experiments. Salivary glands were dissected in physiological saline containing 160 mM NaCl, 10 mM KCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 10 mM glucose, and 10 mM Tris, pH 7.4 as described previously (34). Small lobes consisting of several acini with their corresponding branched duct system were used. In some experiments lobes were superfused with low-$\text{Cl}^-$ physiological saline, in which all NaCl was substituted with equimolar amounts of NaNO$_3$ or Na-isethionate and other $\text{Cl}^-$ salts were substituted with sulphate salts except for 2 mM CaCl$_2$.

Reagents. Solutions of 10 mM dopamine (Sigma-Aldrich, Deisenhofen, Germany) in double-distilled water, 500 $\mu$L 4,4′-diisothiocyanatomethylidihydrostilbene-2,2′-disulfonic acid disodium salt (SBFI, Sigma-Aldrich) was prepared in water. Stock solutions of 10 mM nigericin, 40 mM tributyltin, 100 mM bumetanide (all Sigma-Aldrich), 6 mM monomycin (Axxora, Grünberg, Germany), 2 mM sodium-binding benzoazin isophthalate acetoxymethyl ester (SBFI/AM), and 2 mM Sodium Green tetra-acetate (both from Invitrogen) were prepared in DMSO and stored in small aliquots at $-20^\circ$C. All stock solutions were diluted in physiological saline immediately before an experiment. The final concentration of DMSO did not exceed 0.1% (vol/vol) except for the in situ [Cl$^-$] calibration (0.2%) and the Sodium Green tetra-acetate loading procedure (0.3%).

$\text{Cl}^-$ measurements. $\text{Cl}^-$ recordings in acinar peripheral cells were performed with the $\text{Cl}^-$-sensitive fluorescent dye MQAE (76). Thus isolated lobes were incubated for $\approx$30 min at room temperature in a hypotonic physiological saline (75% physiological saline + 25% double-distilled water) containing 10 mM MQAE. After dye loading, lobes were removed from the loading medium and acclimatized in physiological saline for at least 15 min. MQAE-loaded lobes were then attached to a coverslip coated with the tissue adhesive Vectabond Reagent (Axxora, Grünberg, Germany). The coverslip was fixated on a custom-built recording chamber (volume $\approx 1$ ml) for continuous superfusion with physiological saline at a flow rate of $\approx 2$ ml/min. 2P microscopy in combination with FLIM was carried out by using the MicroTime 200 fluorescence lifetime microscope (PicoQuant, Berlin, Germany). The setup includes an inverted microscope (IX 71; Olympus, Hamburg, Germany) equipped with an Olympus PlanApo ×100/NA 1.4 oil-immersion objective, on which the recording chamber could be mounted. 2P excitation was performed by a mode-locked femtosecond laser fiber laser (C-Fiber A 780; MenloSystems, Martinsried, Germany) operating at 780-nm, 50-MHz pulse repetition rate and 90-fs pulse width. The near-infrared laser beam was guided toward the objective via the microscope side port by using a dichroic mirror (2P-dichroic 725; Chroma, Fürstenfeldbruck, Germany). The fluorescence light was guided through a 100-$\mu$m pinhole and two shortpass filters for total excitation light rejection (1×SP420–680 OD2, Baader, Mannendorf, Germany; 1×SP400–680 OD4; Edmund Optics, Karlruhe, Germany) and was detected with a single-photon avalanche diode (SPCM-AQR-13; Perkin Elmer, Waltham, MA).

Time-resolved fluorescence image acquisition occurred by raster scanning the objective using a xy-piezo-positioner (Physik Instrumente, Karlruhe, Germany) in the time-correlated single-photon counting (TCSPC) mode by using a TimeHarp 200 PC-board (PicoQuant). This board stored the data in the time-tagged time-resolved mode, recording every detected photon with its individual timing in relation to a reference synchronization signal provided by the fiber laser itself. Laser power was adjusted to achieve average photon counting rates of $10^3$–$10^5$ photon/s and peak rates close to $10^6$ photon/s, thus below the maximum counting rate allowed by the TCSPC electronics to avoid pulse pile up. Acquisition time per pixel accounted for 0.8–2.0 ms, resulting in an image overall acquisition time of 30–90 s. Images were acquired at rates of 0.2–2 images per minute. Faster acquisition rates of eight images per minute were realized with the point measurement mode by accumulating the detected photons at one point within an acinar peripheral cell for 6 s. Data acquisition and analysis were performed by the SynPhoTime software (PicoQuant). Briefly, all photons collected in a region of interest covering one acinar peripheral cell were used to form a global histogram for quantification of the mean cellular fluorescence decay time, thereby ignoring possible subcellular distinctions. Fluorescence decay analysis occurred by tail fitting starting 1 ns after peak maximum to avoid an influence of the instrument response function (IRF). The quality of decay fitting was estimated by randomly distributed residuals and reduced $\chi^2$ values. The full width at half-maximum of the daily measured IRF of the 2P-FLIM setup was typically 300–400 ps.
The dependence of the Cl\(^{-}\) concentration on the fluorescence decay time \(\tau\) is described by the Stern-Volmer equation \(n/\tau = 1 + K_{SV}[\mathrm{Cl}^{-}]\), where \(K_{SV}\) is the Stern-Volmer constant indicating the Cl\(^{-}\) sensitivity of MQAE fluorescence (20). To quantify the dependence of the fluorescence decay time on \([\mathrm{Cl}^{-}]\), in MQAE-loaded salivary acinar peripheral cells, in situ calibration experiments were performed by using the double-ionophore technique (41). Bath application of the K\(^{+}\)/H\(^{+}\) exchanger nigericin (10 \(\mu\)M) and the Cl\(^{-}\)/OH\(^{-}\) exchanger tributyltin (40 \(\mu\)M) resulted in the equilibration of extra- and intracellular \([\mathrm{Cl}^{-}]\) while pH was clamped. Calibration solutions of varying \([\mathrm{Cl}^{-}]\) concentrations (0–75 mM) contained 120 mM (KCl + KNO\(_{3}\)), 10 mM NaNO\(_{3}\), 10 mM glucose, 90 mM mannitol, and 10 mM Tris, pH 7.4. Thus a MQAE-loaded salivary lobe was exposed to only one calibration solution for 20 min. After this incubation period, image acquisition of several salivary acini of this salivary lobe occurred within the next 10 min. Stepwise changes in calibration solutions at one salivary lobe were not feasible because cells frequently deteriorated in the nigericin-tributyltin calibration solution.}

\([\mathrm{Na}^{+}]\) measurements. \([\mathrm{Na}^{+}]\) recordings in acinar peripheral cells were performed with the \([\mathrm{Na}^{+}]\)-sensitive fluorescent dyes SBFI and Sodium Green. Isolated lobes were incubated for 1–2 h at room temperature in a hypotonic physiological saline (75% physiological saline + 25% double-distilled water) containing either 3.75 \(\mu\)M SBFI/AM or 6.7 \(\mu\)M Sodium Green tetra-acetate. After dye loading, the further preparation and image recordings of the lobes were performed with the 2P-FLIM setup as described for \([\mathrm{Cl}^{-}]\) measurements with 2P excitation at 780 nm and fluorescence detection at 420–680 nm. Thus lobes were raster scanned (150 \(\times\) 150 pixel) at a rate of 1 per minute, and fluorescence intensity images were calculated by integrating all detected photons per pixel, thereby ignoring the temporal information usually essential for fluorescence-decay-time calculation. Subsequently, regions of interest of the recorded image stacks were analyzed with the MetaMorph 7.1 software (Universal Imaging, Downingtown, PA). Qualitative changes in the \([\mathrm{Na}^{+}]\) concentration are presented as background-corrected fluorescence intensities.

**RESULTS**

Behavior of MQAE in salivary acinar peripheral cells. Salivary acini without dye loading displayed a comparatively low autofluorescence when excited at 780 nm as expected for 2P excitation (Fig. 1C). In contrast, the fluorescence intensity of MQAE-loaded salivary acini was typically more than one order of magnitude higher than that of unloaded acini (Fig. 1, A and B). In addition, the duct epithelium was stained strongly and served as reference because MQAE fluorescence in ducts was studied previously (30). However, the acini were stained weaker and MQAE fluorescence could be detected only at the acinar surface, where the peripheral cells form the base of each acinus. Within the acinar peripheral cells the MQAE fluorescence was not homogeneously distributed. The peripheral cells exhibit a pyramidal shape, but, whereas the cytosol was stained sufficiently, no staining could be observed in the numerous apical long microvilli facing the duct lumen, resulting in goblet-like stained cells.

To determine the relation between \([\mathrm{Cl}^{-}]\) and the MQAE fluorescence decay time, in situ calibration experiments were performed. Loaded into acinar peripheral cells, MQAE showed a biexponential fluorescence decay. A long time component \(\tau_1\) clearly decreased from \(\approx 5.8\) ns to \(\approx 4.2\) ns when increasing \([\mathrm{Cl}^{-}]\) from 0 mM to 75 mM, thereby possessing a constant relative amplitude \(\alpha_1\) of \(\approx 70\%\) (Fig. 1D). A short time component

(see Supplemental Fig. S2; supplemental material for this article is available online at the Am J Physiol Cell Physiol website).

For evaluation of the setup a solution of \(3.0 \times 10^{-8}\) M rhodamine 6G in ethanol was prepared as well. All solutions were stored at \(-4\)°C.

2P fluorescence-excitation-action cross sections \(\Phi_{2P}(\lambda)\) (with \(10^{-50}\) cm\(^4\) s/photon = 1 GM) were calculated according to the equation

\[
\Phi_{2P}(\lambda) = (f_{FD} v)(\Phi_{2S}\tau_{2S} I f_{FD} v),
\]

where \(f_{FD} v\) is the integral of the 2P emission spectrum, \(c\) is the dye concentration, and \(\Phi_{2S}\) is the fluorescence quantum yield (68). The subscript \(S\) indicates the parameters of the fluorescent dye fluorescein, which was used as 2P excitation standard with known \(\Phi_{2S} = 0.93\) and 2P-absorption cross sections \(\sigma_{2S}\) (50, 70). Quantum yields were determined absolutely with the C 9929 integration sphere system (Hamamatsu, Hamamatsu City, Japan). For \(f_{FD} v\) determination a mode-locked Ti:Sa laser system (Tsunami 3960; Spectra Physics, Mountain View, CA) was used as excitation source operating at 82-MHz repetition rate with a pulse width of 80 fs and \(\approx 260\) mW average laser power at 780 nm. The Ti:Sa laser was tuned from 700 nm to 800 nm in 10-nm steps. The laser light was coupled into the fluorescence lifetime spectrometer FL 920 (Edinburgh Instruments, Edinburgh, UK) and focused via a lens on the aqueous solutions of fluorescein and SBFI, respectively. Stability of the average laser power was controlled between measurements.

Data analyses. Nonparametric tests were applied for statistical comparisons attributable to partial nonnormality (D’Agostino-Pearson normality test) coupled with small sample sizes. Thus differences of two data sets within one experiment were analyzed by Wilcoxon matched-pairs tests, whereas differences of data sets between two experiments were analyzed by Mann-Whitney U-tests. Comparisons between more than two independent data sets were calculated by a Kruskal-Wallis H-test followed by Dunn’s multiple-comparison post hoc tests. Differences were considered statistically significant if \(P < 0.05\). Data from the \([\mathrm{Cl}^{-}]\) recovery experiments were fit to the single exponential equation

\[
[\mathrm{Cl}^{-}] = [\mathrm{Cl}^{-}]_{\text{in}} + ([CI]_{\text{in}} - [CI]_{\text{in}}) \cdot \exp(-t/\tau),
\]

where \([\mathrm{Cl}^{-}]_{\text{in}}\) and \([\mathrm{Cl}^{-}]_{\text{in}}\) represent the \([\mathrm{Cl}^{-}]\) at time \(t\) at \(t = 0\) and after full recovery, respectively. From the time constant \(\tau\) the half-time of recovery could be calculated according to \(t_{1/2} = \tau/\ln 2\). Statistical analyses were performed using GraphPad Prism 4.01 (GraphPad Software, San Diego, CA). Graphical presentation was carried out by Origin 7G (OriginLab, Northampton, MA). Data are presented as means \(\pm\) SE.
component of $\tau_2 = 1.0 - 1.1$ ns did not vary because of $[\text{Cl}^-]_i$ changes. According to the equation $\tau_{av} = (\alpha_1 \cdot \tau_1^2 + \alpha_2 \cdot \tau_2^2) / (\alpha_1 \cdot \tau_1 + \alpha_2 \cdot \tau_2)$ (42), the intensity-weighted average fluorescence decay times $\tau_{av}$ from 25–68 different cells of 13–41 acini per Cl$^-$ concentration were calculated as $[\text{Cl}^-]_i$-dependent parameter for further investigations (Fig. 1D, inset). Thus the decay time in the absence of Cl$^-$ was calculated to $\tau_{av,0} = 5.5 \pm 0.1$ ns ($n = 26$ cells). The Stern-Volmer plot of the calibration data yielded a Stern-Volmer constant of $K_{SV} = 5.8 \text{ M}^{-1}$, which was used in all further experiments to calculate $[\text{Cl}^-]_i$, from the recorded MQAE average fluorescence decay times (Fig. 1E). Comparisons of decay analysis methods (tail fit vs. deconvolution fit) as well as of $\text{Cl}^-$-dependent analysis parameters ($\tau_1$ vs. $\tau_{av}$) resulted in a similar $\text{Cl}^-$-dependent behavior of MQAE although the absolute decay times varied between the methods. However, this variation also occurred in physiological measurements, resulting in similar mean resting $[\text{Cl}^-]_i$ values independent of the analysis method (see Supplemental Figs. S1 and S2).

Resting $[\text{Cl}^-]_i$ in salivary acinar peripheral cells. In several preparations $[\text{Cl}^-]_i$ seemed to display slight variations within a peripheral cell under resting conditions although only the mean $[\text{Cl}^-]_i$ of a whole peripheral cell could be reliably evaluated (Fig. 2A). The analysis of 105 cells from 88 different preparations revealed $[\text{Cl}^-]_i$ variations from 18 mM to 83 mM. The values were approximately normally distributed (Fig. 2B), and the mean resting $[\text{Cl}^-]_i$ was 48.9 ± 1.4 mM ($n = 105$ cells). Because $[\text{Cl}^-]_i$ seemed to be somewhat above the $\text{Cl}^-\text{electrochemical equilibrium}$ within the acinar peripheral cells, the activity of $\text{Cl}^-$-dependent transport proteins under resting conditions was studied pharmacologically. Bath application of 50 µM bumetanide, a specific inhibitor of the NKCC (69), seemed to transiently reduce $[\text{Cl}^-]_i$; however, this alteration was not significant (Fig. 2C). The combined bath application of 50 µM bumetanide and 500 µM H2DIDS, a nonspecific inhibitor of the AE in mammalian and insect tissues (7, 8), had clearly no influence on the resting $[\text{Cl}^-]_i$ (Fig. 2D).

NKCC-dependent $\text{Cl}^-$ uptake after bath $\text{Cl}^-$ substitution. When isolated lobes were perfused with low-$\text{Cl}^-$ saline for 30 min, mean $[\text{Cl}^-]_i$ significantly decreased from 47 ± 3 mM to 25 ± 5 mM in acinar peripheral cells (Fig. 3A, $n = 6$, $P < 0.05$). Despite long-time treatment with low-$\text{Cl}^-$ saline, no complete intracellular $\text{Cl}^-$ loss could be observed. $\text{Cl}^-$ substitution with the relatively large, membrane-impermeable anion isethionate could prevent intracellular charge equalization, resulting in diminished intracellular $\text{Cl}^-$ loss. However, $\text{Cl}^-$ substitution with the smaller, membrane-permeable anion NO3$^-\text{ resulted in a }[\text{Cl}^-]_i\text{ decrease to about 20 ± 4 mM }$ (Fig. 3A, $n = 7$), which was not significantly different to that when using isethionate. On the other hand, the effect was reversible because readdition of $\text{Cl}^-$-rich physiological saline caused a rapid increase in $[\text{Cl}^-]_i$ until the initial value was reached after around 5–10 min. Because the $[\text{Cl}^-]_i$ recovery occurred much faster than its foregoing drop, possible pathways for rapid $\text{Cl}^-$ influx during readdition of $\text{Cl}^-$-rich physiological saline were investigated. Because of the limited recorded time points and
the data fluctuations during the recovery periods, the identification of different recovery behavior under several conditions was somewhat hindered (Fig. 3, C and D). Thus recovery half-times $t_{1/2}$ were calculated from single exponential curve fits of the first 12 min of recoveries and used for statistical comparisons although they did not refer to a precise kinetic parameter (Fig. 3B). After readDITION of physiological saline, $[\text{Cl}^-]$, increased with a half-time of $3.1 \pm 0.6$ min ($n = 13$) to reach the initial resting value (Fig. 3, B and C). However, in the presence of $50 \mu \text{M}$ bumetanide, the half-time is significantly increased to $18.9 \pm 4.4$ min ($n = 10$, $P < 0.01$). The combined bath application of $50 \mu \text{M}$ bumetanide and $500 \mu \text{M}$ H$_2$DIDS did not further increase the half-time ($14.8 \pm 3.7$ min, $n = 10$, $P > 0.05$).

Stimulation of isolated lobes by bath application of $1 \mu \text{M}$ dopamine during readDITION of $\text{Cl}^-$-rich physiological saline resulted in a putative shorter recovery half-time of $1.1 \pm 0.3$ min ($n = 12$). Nevertheless, it was not significantly different from the half-time in the absence of dopamine ($P > 0.05$, Fig. 3, B and D). Additional bath application of $50 \mu \text{M}$ bumetanide with or without $500 \mu \text{M}$ H$_2$DIDS resulted in a similar behavior as observed in the absence of dopamine. In detail, bumetanide in the presence of dopamine significantly increased the half-time to $7.7 \pm 1.8$ min ($n = 14$, $P < 0.01$), whereas bumetanide together with H$_2$DIDS did not further increase the half-time ($12.0 \pm 1.7$ min, $n = 8$, $P > 0.05$). These results indicate the contribution of the NKCC to the rapid $\text{Cl}^-$ influx both in the absence and presence of dopamine. However, no accessory contribution of a H$_2$DIDS-sensitive anion transporter could be unraveled.

**Lack of dopamine-induced $[\text{Cl}^-]$, changes.** Stimulation of isolated lobes by bath application of $1 \mu \text{M}$ dopamine, a concentration known to elicit fluid secretion (36) and duct cell activity (44), caused no significant changes in $[\text{Cl}^-]$, of the acinar peripheral cells ($P > 0.05$, Fig. 4A). A second stimulation period revealed the same result although a second dopamine stimulation can result in stronger effects as seen for changes in $[\text{Ca}^{2+}]$, in salivary duct cells (43). In addition to the fluorescence decay time analysis, the MQAE fluorescence intensity was recorded. The fluorescence intensity trace displayed a continuous decrease even under resting conditions, most likely attributable to photobleaching and dye leakage. This $[\text{Cl}^-]$-independent decline was observed to a varying extent among the experiments. However, to correct for this run down and to resolve physiological effects, data points of an experiment that have been obtained in physiological saline were fitted to a single exponential decay function, and subsequently all recorded data points were corrected for this run.
down. However, no significant dopamine-induced shift in the MQAE fluorescence intensity could be observed \( (P > 0.05) \), Fig. 4A. This suggested the absence of pronounced volume changes of the acinar peripheral cells. Nevertheless, slight but inconsistent intensity fluctuations could be recognized during dopamine stimulation, most probably attributable to the overall movement of the isolated lobe during stimulation. The absence of any alteration in \([\text{Cl}^-]\) could be still the result of fast transient \([\text{Cl}^-]\) changes, which cannot be detected when using a data acquisition rate of 1 per minute. Consequently, \([\text{Cl}^-]\) recordings during dopamine stimulation were also performed at an acquisition rate of 8 per minute by recording only the photons of one pixel within the cytosol of a peripheral cell for fluorescence decay analysis. The analysis of the initial 2 min after dopamine bath application resulted in no dopamine-induced \([\text{Cl}^-]\) variations (Fig. 4B). However, continuous irradiation at one point caused strong photobleaching and tissue damages preventing long-term physiological point experiments.

The lack of significant \([\text{Cl}^-]\) changes could be also the result of the simultaneous activities of apical \(\text{Cl}^-\) efflux and basolateral \(\text{Cl}^-\) reuptake mechanisms during dopamine-induced fluid secretion in the acinar peripheral cells. To test this hypothesis, we used the fact that saliva secretion in the cockroach *Nauphoeta cinerea* still happened when extracellular \(\text{Cl}^-\) was replaced by \(\text{NO}_3^-\), whereas use of the larger anion methylsulphate instead of \(\text{NO}_3^-\) did totally abolish secretion \( (71) \). Therefore, when perfusing the isolated lobes with low-
\(\text{Cl}^-\) saline with \(\text{NO}_3^-\) as substitute, one can observe the decrease in \([\text{Cl}^-]\) in the peripheral cells as shown before \( (P < 0.05) \). The analysis of the recovery half-times and the corresponding recovery time traces after readdition of physiological saline (indicated by an arrow) in the absence and presence of dopamine and inhibitors of \(\text{Cl}^-\)-dependent transport proteins; \( n = 4 – 10 \) acini with different numbers of analyzed cells indicated in parentheses in B; means ± SE, Kruskal-Wallis H-test and Dunn’s multiple-comparison post hoc tests, \( **P < 0.01 \).
limited [Na⁺] dependence as found out from preliminary tests and previous studies (11, 14).

SBFI is generally used as a dual-excitation ratiometric probe because its one-photon-excitation peak is shifted to shorter wavelengths upon Na⁺ binding. Thus the SBFI fluorescence ratio \( \frac{F_{340}}{F_{380}} \) after one-photon excitation at 340 nm and 380 nm raises with increasing [Na⁺], allowing quantitative recordings (55). However, in the present study SBFI was 2P excited at only one wavelength (\( \lambda_{ex} = 780 \) nm), which requires knowledge about its Na⁺-dependent behavior at this excitation wavelength. Thus 2P fluorescence excitation action cross sections (\( \Phi_2 \sigma_2 \)) of SBFI were determined by exciting SBFI in vitro (water, pH 7.4) in a range of 700–800 nm in the absence and presence of 125 mM NaCl. The fluorescence quantum efficiencies of the SBFI Na⁺-free form and Na⁺-bound form were determined to \( \Phi_F = 0.06 \pm 0.01 \) (\( n = 5 \)) and \( \Phi_F = 0.10 \pm 0.01 \) (\( n = 3 \)), respectively, which agree with previously published data (55). 2P excitation spectra of SBFI displayed a spectral shift upon Na⁺ binding with an isosbestic point at around 750 nm. \( \Phi_2 \sigma_2 \) of the Na⁺-bound SBFI form is higher than that of the Na⁺-free SBFI form at wavelengths shorter than the isosbestic point and vice versa at longer wavelengths (Fig. 5A). Thus 2P excitation of SBFI at 780 nm as performed in this study should result in a decreasing fluorescence intensity when [Na⁺] increases (at 780 nm: \( \Phi_2 \sigma_2 \), Na⁺-free \( \sim 0.57 \) GM vs. \( \Phi_2 \sigma_2 \), Na⁺-bound \( \sim 0.35 \) GM).

To avoid possible influences of ester hydrolysis step,

limited [Na⁺], dependence as found out from preliminary tests and previous studies (11, 14).

Fig. 4. Effect of dopamine stimulation on [Cl⁻] in salivary acinar peripheral cells. A: comparison of [Cl⁻] data calculated from MQAE fluorescence decay times and the corresponding fluorescence intensities presented as raw and background-corrected fluorescence intensity data; \( n = 13 \) cells of 11 acini, means ± SE. B: point measurements of [Cl⁻], in peripheral cells during dopamine stimulation; \( n = 15 \) cells of 12 acini, means ± SE. C: long-time stimulation with dopamine in the presence of low-Cl⁻ saline, in which NaCl is substituted by NaNO₃; \( n = 6 \) cells of 5 acini, means ± SE, Wilcoxon matched-pairs test, *\( P < 0.05 \).

Fig. 5. 2P fluorescence excitation action cross-section spectra of the Na⁺-free and the Na⁺-bound sodium-binding benzofuran isophthalate tetra-ammonium salt (SBFI) form in aqueous buffer solution (\( n = 3 \)) (A) and in cytosol-like solution (\( n = 2 \), at 700 nm \( n = 1 \)) (B). Fluorescein in NaOH, pH 11, served as reference. The 2P-action cross section is the product of the fluorescence quantum yield \( \Phi_F \) and the absolute 2P absorption cross section \( \sigma_2 \); 1 GM = 10⁻¹⁰ cm²/s/photon.
recordings of the SBFI tetra-ammonium salt were performed with 2P excitation at 800 nm. The results from these experiments [at 800 nm: $\Phi_{2P} \sigma_2, \text{Na}^+-\text{free} \sim 0.47 \text{ GM (n = 3)}$ vs. $\Phi_{2P} \sigma_2, \text{Na}^+-\text{bound} \sim 0.18 \text{ GM (n = 6)}$] fit quite well to the corresponding results on the basis of measurements of hydrolyzed SBFI (at 800 nm: $\Phi_{2P} \sigma_2, \text{Na}^+-\text{free} \sim 0.27 \text{ GM vs. } \Phi_{2P} \sigma_2, \text{Na}^+-\text{bound} \sim 0.12 \text{ GM}$). However, the spectral properties of SBFI can be altered in the intracellular environment, probably because of different viscosity, ionic strength, and protein content (3). This fact emphasizes the importance of the in situ determination of SBFI 2P excitation spectra, but it was technically infeasible to determine 2P fluorescence excitation spectra of SBFI-loaded salivary peripheral cells. Alternatively, spectra were collected in vitro using a cytosol-like buffer solution (pH 7.4), which has been prepared from homogenized salivary glands. Compared with the 2P excitation spectra measured in water, the spectra were somewhat changed because no clear isosbestic point could be observed (Fig. 5A). Nevertheless, 2P excitation of SBFI-loaded cells at 780 nm should clearly result in a decreasing fluorescence intensity when [Na$^+$], increases because the $\Phi_{2P} \sigma_2$ ratio between the Na$^+$-free and Na$^+$-bound form is even more enhanced ($\Phi_{2P} \sigma_2, \text{Na}^+-\text{free} \sim 1.27 \text{ GM vs. } \Phi_{2P} \sigma_2, \text{Na}^+-\text{bound} \sim 0.28 \text{ GM}$). Because of relatively small determined $\Phi_{2P} \sigma_2$ values, control of setup and data analysis occurred by using the 2P standard dye rhodamine 6G. 2P excitation of rhodamine 6G at 800 nm resulted in $\Phi_{2P} \sigma_2 = 35.2 \pm 1.1 \text{ GM (n = 9)}$.

SBFI could be sufficiently loaded into cockroach salivary acini by using a hypotonic shock procedure, which resulted in an intracellular dye distribution as seen for MQAE before. In addition to strongly stained ducts, only peripheral cells at the surface of the acini were stained (cf., MQAE staining in Fig. 1A). For in situ calibration of the SBFI fluorescence intensity related to changes in [Na$^+$], 0–100 mM Na$^+$ calibration solutions with different combinations of gramicidin (Na$^+$ ionophore), monensin (NHE), and nigericin (K$^+$/H$^+$ exchanger) were tested. However, no reliable and consistent results could be obtained, most likely because of insufficient dissipation of the Na$^+$ gradient across the plasma membrane. Thus the following SBFI experiments were only qualitatively analyzed in terms of decrease vs. increase in [Na$^+$].

A drawback of measuring fluorescence intensities at one excitation wavelength as performed with the dye SBFI in this study is the insufficient discrimination between fluorescence intensity changes attributable to variations in the respective ion concentration and changes attributable to cell volume changes or dye leakage and photobleaching. Assuming a constant dye leakage and/or photobleaching rate during a whole experiment, the raw data could be fairly corrected for this. However, stimulus-induced cell volume changes cannot be predicted or excluded a priori. Thus, to confirm that stimulus-induced changes in the SBFI fluorescence intensity correspond rather to [Na$^+$] changes than to cell volume effects in acinar peripheral cells, [Na$^+$] recordings were also performed with the Na$^+$-sensitive fluorescent dye Sodium Green. In contrast to SBFI, Sodium Green is a single-excitation nonratiometric probe, and its fluorescence intensity rises because of Na$^+$ binding when excited at around 488 nm without any shift in the excitation spectrum in vitro and in situ (1). Thus 2P excitation of Sodium Green should definitively result in a fluorescence intensity increase attributable to a [Na$^+$] rise independent of the 2P excitation wavelength. Lower loading efficiency into the acinar peripheral cells of Sodium Green has required higher dye concentrations, but intracellular dye distribution was similar to that observed with SBFI. As seen for SBFI, in situ calibration of the Sodium Green fluorescence intensity was not feasible and indicated that the reason for this problem is rather insufficient dissipation of the Na$^+$ gradient across the plasma membrane than the dye itself.

Dopamine-induced [Na$^+$], rise. One would expect no changes in [Na$^+$], under resting conditions in the presence of physiological saline, but the fluorescence intensity time traces of SBFI and Sodium Green exhibited a continuous [Na$^+$]-independent decrease (Fig. 6). Thus the fluorescence-intensity data were corrected for this run down as performed for the MQAE recordings (see Fig. 4A).

Bath application of 1 nM dopamine caused a rapid increase in [Na$^+$], in salivary acinar peripheral cells. This fact could be concluded from the opposite behavior of both Na$^+$-sensitive fluorescent dyes attributable to dopamine stimulation. Whereas the SBFI fluorescence intensity increased rapidly by about 10% in acinar peripheral cells (n = 33, P < 0.001, Fig. 6A), the Sodium Green fluorescence intensity increased by about 10% (n = 22, P < 0.001, Fig. 6B). When we used SBFI, the [Na$^+$], rise recovered essentially within 5 min after dopamine removal. In addition, a second dopamine stimulation after 25 min caused again a reversible [Na$^+$], rise but to a lower extent, however, indicating the viability of the isolated lobes (P < 0.001, Fig. 6A). On the other hand, the dopamine-induced [Na$^+$], rise measured with Sodium Green recovered essentially slower, and no effect of a subsequent second dopamine stimulation could be recognized (Fig. 6B). Because the repeated SBFI recordings appeared to be reliable, Sodium Green seemed to respond differently after an initial treatment, maybe because of an interference with cellular components. Thus the following experiments were performed predominantly with SBFI, and from additional Sodium Green recordings only the response after an initial treatment was analyzed.

The effect of dopamine on [Na$^+$], was also studied in salivary duct cells to get a feeling of the direction and magnitude of the SBFI fluorescence intensity changes. Repeated bath application of 1 nM dopamine caused a reversible intensity decline by about 13% and 7% in salivary duct cells, respectively, as well suggesting a [Na$^+$], rise (n = 8, P < 0.01 and P < 0.05, Fig. 6C). Indeed, dopamine stimulation has been previously shown to induce a huge reversible intracellular Na$^+$ elevation in duct cells from 22 mM to 92 mM by using Na$^+$-sensitive microelectrodes (44). Thus a small change in the SBFI fluorescence intensity can still correspond to a large change in [Na$^+$].

Involvement of NKCC in dopamine-induced [Na$^+$], rise. The NKCC is thought to be one major basolateral NaCl-uptake mechanism in the salivary acini, and it could be responsible for stimulus-induced [Na$^+$] alterations. Indeed, the dopamine-induced [Na$^+$], rise in the acinar peripheral cells was Cl$^-$ dependent because it could be reversibly suppressed when perfusing the isolated lobes with low-CI$^-$ saline (n = 8, P > 0.05, Fig. 7A). In addition, bath application of 50 nM bumetanide alone did not influence the resting [Na$^+$], but mostly abolished its dopamine-induced increase in acinar peripheral cells (n = 28, P < 0.05, Fig. 7B). This inhibitory effect was reversible because a second control stimulation with dopamine
in the absence of bumetanide resulted again in a [Na\(^+\)]\(_i\) rise when using SBFI. When we used Sodium Green, bath application of 50 \(\mu\)M bumetanide, not only abolished the dopamine-induced [Na\(^+\)]\(_i\) increase in salivary peripheral cells, but also resulted in a reversible [Na\(^+\)]\(_i\) decrease (\(n = 20\), \(P < 0.001\), Fig. 7C).

An increase in the intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) is generally thought to be the primary fluid secretion signal in salivary acinar cells. Thus the effect of an artificially induced [Ca\(^{2+}\)]\(_i\) rise on [Na\(^+\)]\(_i\), in salivary acinar peripheral cells was investigated. Bath application of 3 \(\mu\)M ionomycin, a Ca\(^{2+}\) ionophore known to elevate intracellular Ca\(^{2+}\) in cockroach salivary gland cells (59), caused an irreversible [Na\(^+\)]\(_i\) rise in the peripheral cells, which was significantly larger than that induced by a previous dopamine stimulation (\(n = 22\), \(P < 0.05\), Fig. 7D). However, this ionomycin-induced [Na\(^+\)]\(_i\) rise could be significantly diminished by bath application of 50 \(\mu\)M bumetanide (\(n = 25\), \(P < 0.01\), Fig. 7D).

**DISCUSSION**

Although the salivary glands of the cockroach *P. americana* are a well-established model system for studying epithelial transport and aminergic signal transduction, so far no intracellular ion recordings could be successfully performed in living acinar cells mainly as a result of methodological problems. In this study we present for the first time intracellular Na\(^+\) and Cl\(^-\) recordings from living acinar peripheral cells of isolated cockroach salivary glands. In fact, the performance of the innovative microscopic technique 2P-FLIM gained access to the ion homeostasis in salivary glands, which advances the existing model of saliva secretion.

Quantitative 2P-FLIM [Cl\(^-\)], measurements were performed with the Cl\(^-\)-sensitive fluorescent dye MQAE. It was well suited for 2P excitation at 780 nm when loaded into the salivary peripheral cells and displayed a sufficient dynamic range of its fluorescence decay time changes in response to variations in [Cl\(^-\)]\(_i\), as known from previous studies (30, 37). The in situ calibration resulted in a slightly lower MQAE sensitivity to Cl\(^-\) in peripheral cells than in duct cells (30). This demonstrates the importance of separate calibration experiments in different cell types although they are located within the same tissue and serve for a similar function. However, the \(K_{SV}\) value of 5.8 M\(^{-1}\) fits well to previously reported \(K_{SV}\) values in other cell types in the range of 3–26 M\(^{-1}\) (21, 46, 49). The most critical point of the in situ [Cl\(^-\)]\(_i\) calibration is the application of the toxic Cl\(^-\)/OH\(^-\) exchanger tributyltin, which can influence cellular physiology at higher concentrations (19). Therefore, for satisfactory calibration procedure, each isolated lobe has been treated with only one calibration solution. Despite this problem, one advantage of using FLIM is that \(K_{SV}\) is only tissue dependent but does not vary between the preparations of one particular tissue, simplifying the calibration procedure.

On the other hand, FLIM [Na\(^+\)], measurements are rather challenging. The fluorescence decays of SBFI and Sodium Green display a complex triexponential decay behavior in living cells, and their fluorescence decay times or rather corresponding amplitudes are only weakly Na\(^+\) dependent (11, 14, 73, 74). Nevertheless, the present study showed that these dyes are still well suited for fluorescence intensity based 2P microscopy. Although no in situ calibration was possible for the two Na\(^+\)-sensitive dyes SBFI and Sodium Green, useful information was gained from relative fluorescence intensity changes. Both dyes were already tested for 2P excitation in the
excitation wavelength range of 700–940 nm (2, 13, 40, 63). On the one hand, Sodium Green shows an increase in the fluorescence intensity upon Na⁺/H⁺ binding without significant spectral shifts (73). Thus 2P excitation of Sodium Green definitively results in a fluorescence intensity increase attributable to a [Na⁺/H⁺]i rise independent of the 2P excitation wavelength. On the other hand, excitation spectra of SBFI are shifted to shorter wavelengths upon Na⁺/H⁺ binding, resulting in a decrease or increase in the fluorescence intensity attributable to a [Na⁺/H⁺]i rise depending on the excitation wavelength (74). 2P fluorescence excitation spectra of SBFI in vitro and in salivary gland homogenates revealed that the 2P action cross section of the Na⁺/H⁺-bound form was lower than that of the Na⁺/H⁺-free form at 780 nm, the 2P excitation wavelength used in this study. From this result one could consider a decrease in the SBFI fluorescence intensity attributable to a [Na⁺/H⁺]i rise after 2P excitation at 780 nm. This opposed behavior compared with that of Sodium Green could further unravel possible cellular volume effects as performed with the classical ratiometric approach. A qualitatively similar fluorescence behavior of the SBFI Na⁺-bound and Na⁺-free form after 2P excitation was reported previously (2, 13, 63). However, the only reported 2P action cross section of SBFI is that of the Na⁺-bound SBFI tetra-ammonium salt with \( \Phi_{2P,780\text{nm}} = 20 \text{ GM} \) (40). By using both the ester and tetra-ammonium salt of SBFI, possible influences of the ester hydrolysis step could be excluded. Furthermore, the 2P action cross section of the reference dye rhodamine 6G in ethanol was determined to 35 GM. This is akin to the reported value of \( \Phi_{2P,800\text{nm}} = 36 \text{ GM} \) (38) and corresponds well to previous reports of rhodamine 6G in methanol of \( \Phi_{2P,798–806\text{nm}} = 12–126 \text{ GM} \) depending on the applied method (Ref. 50 and references therein). This indicates the correct use of the setup and the data analysis. Despite the unclear discrepancy we used the values determined here because we were mainly interested in the relative 2P-cross-section differences of the Na⁺-bound and Na⁺-free SBFI form.

The mean resting [Cl⁻]i of cockroach salivary acinar peripheral cells could be quantified to 49 mM. The resting [Cl⁻]i in peripheral cells of cockroach salivary glands was so far only determined in frozen-dried cryosections by using electron probe X-ray microanalysis [39 mM, when extracellular Cl⁻ concentration ([Cl⁻]o) was 135 mM], and this fits well with the value presented here (49 mM, when [Cl⁻]o was 174 mM) (24). The resting [Cl⁻]i in acinar peripheral cells was about 1.6

Fig. 7. Pharmacological experiments on the dopamine-induced [Na⁺]i rise in salivary acinar peripheral cells. A: effect of low-Cl⁻ saline perfusion (n = 8 cells of 4 acini) shown compared with the control experiment in physiological saline from Fig. 6A. B and C: effect of bath application of bumetanide using SBFI (n = 28 cells of 18 acini) and Sodium Green (n = 20 cells of 10 acini), each shown compared with the control experiment in physiological saline from Fig. 6. A and B. D: effect of bath application of ionomycin alone (n = 22 cells of 8 acini) and in combination with bumetanide (n = 25 cells of 12 acini). Values are means ± SE, Mann-Whitney U-test (*P < 0.05, **P < 0.01, ***P < 0.001) and Wilcoxon matched-pairs test in D (*P < 0.05).
times higher than predicted from the Cl$^{-}$ electrochemical equilibrium calculated from the Nernst equation (49 mM vs. predicted 29 mM) when assuming a basolateral membrane potential of $-45$ mV (66). In contrast, previous studies in mammalian salivary acinar cells have reported resting [Cl$^{-}$], values ranging from 43–62 mM (when [Cl$^{-}$]o was $\sim 120$ mM), which were four to five times higher than expected from passive Cl$^{-}$ distribution, strongly indicating active Cl$^{-}$ accumulation (18, 45, 57, 78). In agreement with high [Cl$^{-}$], values, the involvement of common Cl$^{-}$-dependent transport proteins such as NKCC and AE in keeping intracellular Cl$^{-}$ high could be demonstrated in these glands (e.g., Ref. 78). In cockroach salivary acinar peripheral cells, no activity of possible Cl$^{-}$ uptake routes via NKCC and AE could be recognized pharmaceutically, which is consistent with the roughly passive Cl$^{-}$ distribution. The long-term drop of extracellular Cl$^{-}$ from 174 mM to 2 mM also showed the relatively low basolateral Cl$^{-}$ permeability in the acinar peripheral cells because [Cl$^{-}$]o declined only to about 20 mM but not to zero. At this, it was irrelevant whether extracellular Cl$^{-}$ was substituted with a membrane-impermeable anion or -permeable anion to compensate for intracellular net positive charges attributable to Cl$^{-}$ efflux. However, after readdition of extracellular Cl$^{-}$, a rapid bumetanide-sensitive, but no additive H$_2$DIDS-sensitive, Cl$^{-}$ uptake could be recognized. Interestingly, Cl$^{-}$ recovery was independent of a dopamine stimulation. Thus the chemical gradients for Na$^+$, K$^+$, and Cl$^{-}$ under these conditions seemed to activate NKCC-mediated Cl$^{-}$ uptake. The role of [Cl$^{-}$]i, in regulation of NKCC activity during secretion in secretory epithelia is well known. At this, a decrease in [Cl$^{-}$]i, reduces a [Cl$^{-}$]-mediated inhibition of the NKCC possibly by enhanced NKCC phosphorylation (25, 62).

Dopamine is known to stimulate the secretion of a NaCl-rich saliva in isolated cockroach salivary glands (36). However, dopamine did not induce [Cl$^{-}$], changes in the acinar peripheral cells under normal physiological conditions. Neither fluorescence decay times nor fluorescence intensities of MQAE altered upon dopamine stimulation. In contrast, quantitative electron-probe X-ray microanalysis of cockroach salivary gland cryosections has indicated a [Cl$^{-}$], rise from 39 mM to 58 mM (when [Cl$^{-}$]o was 135 mM) in acinar peripheral cells after 15–30 min of dopamine stimulation (24). Nevertheless, previous studies revealed quite variable effects of salivary acinar cell stimulation on [Cl$^{-}$], such as a decrease, an increase, or even no change (Ref. 9 and references therein). In salivary acinar cells of some mammalian species, a stimulus-induced transient [Cl$^{-}$], decrease could be observed within the first 10–30 s, which recovered over 2–5 min (17, 47, 53). Such fast transient [Cl$^{-}$], changes could be masked in the present study because of the technically limited acquisition rate of 2 per minute although at least part of the recovery after transient [Cl$^{-}$], changes of this time regime had to be detectable with the setup. However, the performance of rapid point measurements did not confirm the presence of fast transient [Cl$^{-}$], changes in the salivary acinar peripheral cells. Furthermore, the simultaneous basolateral Cl$^{-}$ uptake and apical Cl$^{-}$ efflux at similar rates could also mask [Cl$^{-}$], alterations. However, this scenario was ruled out because the suppression of basolateral Cl$^{-}$ uptake during dopamine stimulation did not cause any [Cl$^{-}$], changes in the salivary acinar peripheral cells.

In contrast, dopamine stimulation of salivary acinar peripheral cells clearly resulted in a [Na$^{+}$], rise as observed from fluorescence intensity measurements after 2P excitation. A stimulus-induced [Na$^{+}$], rise in acinar cells was previously reported for cockroach salivary gland cryosections (from 10 mM to 25 mM) as well as for mammalian salivary glands (15, 24, 47, 62). However, a stimulus-induced transient cell shrinkage to 70–90% of the prestimulation cell volume has been demonstrated in mammalian acinar cells, which is temporally coupled to a transient [Cl$^{-}$], decrease in those cells (18, 56). Because of the opposite behavior of both Na$^{+}$-sensitive dyes used in this study, we could exclude the existence of significant dopamine-induced cell volume changes. Otherwise, one had expected fluorescence changes in one and the same direction for both dyes, e.g., a fluorescence increase attributable to cell shrinkage. Equal relative changes in the fluorescence intensities for both dyes during dopamine stimulation of about 10% also support the conclusion that these changes correspond nearly completely to the [Na$^{+}$], rise. This is based on similar dissociation constants $K_d$ in situ and comparable changes in the fluorescence intensity from the Na$^{+}$-free to the Na$^{+}$-bound form (1, 13, 48). Nevertheless, both dyes also showed differences in their behavior attributable to dopamine stimulation because the Sodium Green fluorescence increase recovered essentially slower and disallowed a second stimulation. Because the repeated SBFI recordings appeared to be reliable, Sodium Green seemed to respond differently after an initial treatment, maybe attributable to an interference with cellular components. Such a different behavior was already reported for other tissues (12, 47).

The dopamine-induced [Na$^{+}$], rise in acinar peripheral cells was clearly dependent on extracellular Cl$^{-}$ and was abolished by bumetanide. These results suggest that the [Na$^{+}$], rise attributable to dopamine stimulation is mediated by a basolaterally located NKCC. Bath application of bumetanide has been previously shown to reduce the dopamine-induced fluid secretion by 92% and the concentrations of Na$^{+}$, K$^+$, and Cl$^{-}$ in the final saliva (60). In addition, bumetanide also influenced the saliva modification in the duct cells, most likely indirectly because of missing primary saliva in the duct lumen (28, 44). From these observations it was concluded that a basolateral NKCC in the acinar peripheral cells is activated for NaCl uptake during dopamine-induced NaCl-rich primary saliva secretion. In this study, the presence of a NKCC in cockroach salivary acinar peripheral cells has been demonstrated for the first time directly by pharmacological experiments in these cells. Therefore, the NKCC seems to be inactive under resting conditions, but it becomes active during stimulation of saliva secretion. Although the NKCC activity in mammalian salivary acinar cells was found to be quite variable, it is a major NaCl-uptake mechanism (75). However, in the presence of HCO$_3^-$/CO$_2$-buffered saline, NKCC activity can be at least partly compensated by paired NHE/AE activity keeping resting [Cl$^{-}$], high, which is not the case in HCO$_3^-$-free saline as used in this study (47). The HCO$_3^-$-dependent NKCC and NHE/AE activity has been also demonstrated indirectly by pH recordings in cockroach salivary duct cells (28). However, to our knowledge no data are available for NKCC regulation in insect salivary glands. On the other hand, ion transport mechanisms in Malpighian tubules, part of the
excretory system in insects, have been studied comprehensively. Thus low NKCC activity in unstimulated tubules was observed for instance in the yellow fever mosquito *Aedes aegypti* and the blood-sucking bug *Rhodnius prolixus*, whereas NKCC activity is dramatically increased during secretion stimulation via cAMP (26, 32).

For mammalian salivary acinar cells it is thought that the transepithelial Cl\(^{-}\) transport provides the driving force for fluid secretion. Basolateral Cl\(^{-}\) uptake mechanisms such as NKCC or paired AE/NHE accumulate intracellular Cl\(^{-}\) above its electrochemical equilibrium, which is required for apical Cl\(^{-}\) efflux via CI\(^{-}\) channels (e.g., 45, 47, 53, 78). The apical Cl\(^{-}\) efflux causes a lumen-negative potential difference, which drives the paracellular Na\(^{+}\) transport into the lumen. Water follows because of the transepithelial osmotic gradient, resulting in an isosmotic NaCl-rich primary saliva. Stimulation of acinar cells also results in a [Na\(^{+}\)]\(i\) rise, which is mainly mediated by Na\(^{+}\)-dependent basolateral Cl\(^{-}\) uptake mechanisms. Elevated [Na\(^{+}\)]\(i\), activates the basolateral Na\(^{+}\)-K\(^{+}\)-ATPase, which in turn extrudes Na\(^{+}\) from the cells maintaining the electrochemical gradient for basolateral K\(^{+}\) and Cl\(^{-}\) reuptake (15, 47, 79). Consequently, mammalian salivary acinar cells rely on the same ion transport mechanisms, which seems to be different in cockroach salivary acini. We suggest that in the cockroach acinar peripheral cells the transepithelial Na\(^{+}\) transport provides the driving force for fluid secretion, which is energized by an apical Na\(^{+}\)-K\(^{+}\)-ATPase. Thus a NKCC would then function as basolateral Na\(^{+}\) uptake mechanism (Fig. 8). Intuitively, a dopamine-induced NKCC activity would result in increased intracellular Na\(^{+}\), K\(^{+}\), and Cl\(^{-}\) concentrations. Because no dopamine-induced [Cl\(^{-}\)]\(i\), changes could be observed, one has to predict an efficient basolateral Cl\(^{-}\) recycling mechanism. Dopamine stimulation has been shown to cause biphasic changes of the basolateral membrane potential in the acinar peripheral cells (66). An initial hyperpolarization seemed to be linked to Ca\(^{2+}\)-activated K\(^{+}\) channels providing a K\(^{+}\) recycling mechanism, whereas the subsequent depolarization was bumetanide sensitive as shown recently (64). Because the NKCC is an electrically neutral cotransporter (69), the NKCC-mediated depolarization of the basolateral membrane can be only understood, when assuming a basolateral Cl\(^{-}\) recycling simultaneously to the hyperpolarization-related K\(^{+}\) recycling combined with an intracellular Na\(^{+}\) accumulation. Indeed, this predicted change in [Cl\(^{-}\)]\(i\), and [Na\(^{+}\)]\(i\), could be demonstrated in the present study. Cl\(^{-}\) channels and a K\(^{+}\)-Cl\(^{-}\) cotransporter could be possible Cl\(^{-}\) recycling mechanisms because they were found in the basolateral membrane of acinar cells (52, 67). Furthermore, it seems unlikely that Cl\(^{-}\) is transported across the acinar peripheral cells during dopamine stimulation because of [Cl\(^{-}\)], being only slightly above its electrochemical equilibrium and suggested basolateral Cl\(^{-}\) recycling. The proposed model favors a paracellular route of acinar Cl\(^{-}\) transport although it remains speculative (Fig. 8).

A stimulus-induced [Ca\(^{2+}\)]\(i\), elevation is generally thought to be the primary fluid secretion signal in salivary acinar cells (54). The knowledge about the second messengers involved in saliva secretion in *P. americana* salivary glands is still limited. The investigation of the pharmacological properties of the dopamine receptor(s) in these glands could not clearly define which dopamine receptor subtypes are present (51). Furthermore, there are diverse reports on the effect of possible second messengers on saliva secretion in the cockroach *N. cinerea*, suggesting the involvement of cAMP and/or [Ca\(^{2+}\)]\(i\) (22, 23). In the present study, a bumetanide-sensitive [Na\(^{+}\)]\(i\), rise could be induced by ionomycin in cockroach salivary acinar peripheral cells. This fact supports the involvement of a [Ca\(^{2+}\)]\(i\), elevation in the NKCC-mediated [Na\(^{+}\)]\(i\), rise (Fig. 8). An ionomycin-induced [Na\(^{+}\)]\(i\), rise could be also observed in rat sublingual acinar cells (79). A [Ca\(^{2+}\)]\(i\), rise can upregulate NKCC in mammalian salivary acinar cells as a result of NKCC phosphorylation, for example, via the cytochrome P450 pathway of the arachidonic acid metabolism (16). Elevated [Ca\(^{2+}\)]\(i\), also effects Ca\(^{2+}\)-activated K\(^{+}\) and Cl\(^{-}\) channels in acinar cells. Thus the altered electrochemical gradients or cell shrinkage that accompanies activation of these ion channels may then trigger NKCC activity (62). However, the mechanisms of interaction between [Ca\(^{2+}\)]\(i\), and NKCC activity in cockroach salivary glands remains speculative. Thus [Ca\(^{2+}\)]\(i\), recordings in the cockroach salivary acinar cells seem to be promising, but they are technically challenging within the acini as already mentioned. However, as shown in this study, 2P microscopy and FLIM can deal with some of these problems, and thus they are powerful techniques to give new insights into physiological research.

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**DISCLOSURES**

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