The role of dye affinity in optical measurements of Ca\(^{2+}\) transients in cardiac muscle

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Kong W, Fast VG. The role of dye affinity in optical measurements of Ca\(^{2+}\) transients in cardiac muscle. Am J Physiol Heart Circ Physiol 307: H73–H79, 2014. First published May 2, 2014; doi:10.1152/ajpheart.00751.2013.—Previous experiments in cultures of neonatal rat myocytes demonstrated that the shape of Ca\(^{2+}\) transients measured using high-affinity Ca\(^{2+}\) sensitive dyes may be misrepresented. The purpose of this study was to examine the role of dye affinity in Ca\(^{2+}\) measurements in intact adult cardiac tissue by comparing optical recordings obtained with high- and low-affinity dyes. Experiments were carried out in porcine left ventricular (LV) wedge preparations stained locally by intramural injection via microcapillaries (diameter = 150 μm) with a low-affinity Ca\(^{2+}\)-sensitive dye Fluo-4FF or Fluo-2LA (nominal K\(_d\) = 7–10 μmol/l), high-affinity dye Rhod-2 (K\(_d\) = 0.57 μmol/l), and Fluo-4 or Fluo-2MA (K\(_d\) < 0.4 μmol/l); in addition, tissue was stained with transmembrane potential (V\(_m\))-sensitive dye RH-237. Optical recordings of V\(_m\) and Ca\(^{2+}\) transient shapes were made using optical fibers (diameter = 325 μm) glued with the microcapillaries. The durations of Ca\(^{2+}\) transients measured at 50% level of recovery (CaD\(_{50}\)) using high-affinity Fluo-4/Fluo-2MA dyes were up to \(\sim 81\%\) longer than those measured with low-affinity Fluo-4FF/Fluo-2LA at long pacing cycle lengths (CL). In Fluo-4/Fluo-2MA measurements at long CLs, Ca\(^{2+}\) transients often (\(\sim 50\%\) of cases) exhibited slow upstroke rise and extended plateau. In Rhod-2 measurements, CaD\(_{50}\) was moderately longer (up to \(\sim 35\%\) ) than in Fluo-4FF recordings, but Ca\(^{2+}\) transient shapes were similar. In all series of measurements, mean action potential duration values were not significantly different (\(P > 0.05\)). The delays between V\(_m\) and Ca\(^{2+}\) upstrokes were comparable for low- and high-affinity dyes (\(P > 0.05\)). In conclusion, measurements of Ca\(^{2+}\) transient in ventricular myocardium are strongly affected by the affinity of Ca\(^{2+}\) dyes. The high-affinity dyes may overestimate the duration and alter the shape of Ca\(^{2+}\) transients.

intracellular calcium transient; optical mapping; optical fiber; dye affinity

CHANGES IN INTRACELLULAR CONCENTRATION of calcium ions (Ca\(^{2+}\)) play important roles in myocardial function including regulation of contractility, electrical excitation, and arrhythmogenesis. Optical imaging with fluorescent dyes is presently the only available method for measurements of rapid Ca\(^{2+}\) changes during cardiac transients, spontaneous oscillations, and waves. Optical Ca\(^{2+}\) imaging can be combined with simultaneous imaging of transmembrane potential (V\(_m\)) allowing studying interaction between V\(_m\) and Ca\(^{2+}\), which is crucial for understanding mechanisms of ECG alternans (2, 9, 11, 21) and cardiac arrhythmias (1, 12, 16, 18, 20, 22, 25, 28). An important requirement in such measurements is faithful reproduction of Ca\(^{2+}\) changes by fluorescent dyes. Previous studies in cultures of neonatal rat myocytes demonstrated that the shape of optical Ca\(^{2+}\) transients (CaT) can be strongly affected by the affinity of Ca\(^{2+}\)-sensitive dyes (5, 6). Measurements that use high-affinity dyes such as Fluo-3, Fluo-4, and Rhod-2 (nominal K\(_d\) = 0.345, 0.35, and 0.57 μmol/l, respectively), which were often used in whole cardiac tissue (3, 4, 14, 15, 17, 18, 24), exhibited CaTs with a duration (CaD) approximately twice as long as action potential duration (APD) and significantly longer than CaDs measured using low-affinity dyes such as Fluo-4FF and Rhod-FF (K\(_d\) = 9.7 and 19 μmol/l, respectively) (5, 6). Mathematical modeling of ion-dye binding showed that this difference could be explained by the nonlinear response of high-affinity dyes to Ca\(^{2+}\) changes (13). Whether this effect applies to the intact adult myocardium is not known. The ionic properties and Ca\(^{2+}\) dynamics in adult and neonatal cells are different, which may result in a different relationship between Ca\(^{2+}\) changes and optical signals. Therefore, the goal of this study was to examine the role of dye affinity in measurements of Ca\(^{2+}\) in the whole myocardium by comparing optical recordings obtained with high-affinity and low-affinity fluorescent dyes.

METHODS

Heart preparation. Experiments were performed in coronary-perfused left ventricle (LV) wedge preparations isolated from porcine hearts. Animal use conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No.85-23, revised 1996). The protocol was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Pigs were anesthetized with telazol (4.4 mg/kg), xylazine (4.4 mg/kg), and antipone (0.04 mg/kg). Anesthesia was maintained with inhalation of isoflurane (1.3–2.5%) in oxygen during surgery. Heparin (500 unit/kg) was given 10 min before heart extraction. To improve heart preservation, cold cardioplegic solution containing (in mmol/l) 110 NaCl, 16 KCl, 16 MgCl\(_2\), 1.2 CaCl\(_2\), and 10 NaHCO\(_3\) was infused into the clamped aorta before excising the heart, and then the coronary arteries were flushed with cardioplegic solution immediately after the heart was removed. A wedge preparation with dimensions of \(\sim 2 \times 5 \text{ cm}^2\) was cut out from anterior LV wall. A branch of the left descending coronary artery was canulated and perfused using a peristaltic pump with Tyrode’s solution containing (in mmol/l) 128.5 NaCl, 4.7 KCl, 0.7 MgCl\(_2\), 0.5 NaH\(_2\)PO\(_4\), 1.5 CaCl\(_2\), 28 NaHCO\(_3\), and 5 or 20 glucose bubbled with 95% O\(_2\) and 5% CO\(_2\) at a temperature of \(\sim 37^\circ\text{C}\). The perfusion pressure was maintained at \(\sim 70\) mmHg.

To prevent motion artifact in optical recordings, muscle contraction was inhibited with 20 μmol/l of blebbistatin (Tocris Bioscience, Ellisville, MO). Since blebbistatin is light sensitive, preparation of blebbistatin solution and all experiments were performed in dark conditions. In addition, Tyrode’s solution was supplemented with 1 μmol/l of anion transporter inhibitor probenecid (Sigma, St. Louis, MO) to prevent active extrusion of Fluo dyes from cells (6).

Dye staining. Previous measurements of Ca\(^{2+}\) in whole cardiac tissue used high-affinity Ca\(^{2+}\)-sensitive dyes delivered via coronary infusion. Preliminary experiments performed in this study demonstrated that this staining method was not successful for low-affinity dyes Fluo-4FF and

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In addition to Ca\(^{2+}\)-sensitive dye staining, the tissue was stained with a \(V_m\)-sensitive dye RH-237 (Biotium, Hayward, CA). This dye was either injected at a concentration of 5 \(\mu\)mol/l via microcapillaries simultaneously with Ca\(^{2+}\)-sensitive dyes or it was delivered before Ca\(^{2+}\) dye staining as a 5-ml bolus of 300-\(\mu\)mol/l dye solution in the bubble trap. Optical measurements started ~12 min after dye injection and continued for up to ~1.5 h.

Optical recordings of Ca\(^{2+}\) and \(V_m\). Optical recordings were performed using silicon fibers with an outer diameter of 325 \(\mu\)m, core diameter of 300 \(\mu\)m, and numerical aperture of 0.39 (FT-300-UMT; Thorlabs, Newtown, NJ). At one end, the fibers were glued to dye injection microcapillaries. At the other end, fibers were flat-cleaved, polished, and mounted on inverted epi-fluorescent microscope (Zeiss Axiosvert 135AV). Several fibers were bundled together and imaged simultaneously. Plastic jackets encompassing individual fibers ensured the lack of inter-fiber cross talk.

Figure 1 shows a schematic diagram of the optical setup and two microcapillary-fiber bundles inserted into LV wedge preparation. Excitation light was provided by a 200-W Hg/Xe lamp. The duration of excitation light exposure was controlled using an electromechanical shutter. To reduce dye photobleaching and possible degradation of blebbistatin by light, the duration of light exposure in each recording was typically limited to 1.2 s. The excitation light was focused on fiber ends using a 10\(\times\) objective lens (Fluar; Carl Zeiss, Thornwood, NY). The same lens was used to collect fluorescent light and focus it on a 16\(\times\)16 photodiode array (C4675-102; Hamamatsu, Japan). Detected optical signals were conditioned and sampled using a previously described mapping system (5) at a sample rate of 1 kHz. With the 10\(\times\) lens, each fiber was projected onto at least four photodiodes. Signals from these photodiodes were averaged to improve the signal-to-noise ratio.

\(V_m\) and Ca\(^{2+}\)-sensitive measurements were performed sequentially by changing optical filter sets. To measure fluorescence of Fluo dyes a filter set included a 480/40-nm excitation filter, a 535/50-nm emission filter, and a 505-nm dichroic mirror. Measurements of Rhod dyes used a 530/40-nm excitation filter, a 580/40-nm emission filter, and a 553-nm dichroic mirror. The RH-237 filter set included a 560/55-nm excitation filter, >720-nm emission filter, and a 600-nm dichroic mirror.

To check for cross talk between \(V_m\) and Ca\(^{2+}\) measurements, optical recordings were performed first by staining the tissue with only one dye (either Ca\(^{2+}\)-sensitive dye or RH-237). Cross talk between Fluo dyes and RH-237 was undetectable. Cross talk between Rhod-2 and RH-237 was below the noise level.

Wedge preparations were stimulated via a bipolar electrode. Action potentials (APs) and CaTs were first recorded at a regular pacing cycle length (CL) of 500 ms and then repeated at CL of 1,000, 2,000, 300, and 250 ms. At the end of this series, measurements were repeated at the 500-ms CL to check for measurements reproducibility. The shapes of APs and CaTs measured at the 500-ms CL before and after the series were similar. To avoid possible motion artifact caused by pulsatile flow, the perfusion pump was stopped during the recording period. Optical Ca\(^{2+}\) recordings exhibited gradual decline of signal magnitude likely due to combination of dye photobleaching and washout. Because of this signal decline, optical recordings taken within 30 min after the start of measurements were used in data analysis.

### Table 1. Nominal dye \(K_d\) values and CaT parameters measured at selected cycle lengths

<table>
<thead>
<tr>
<th>Dye</th>
<th>(K_d), (\mu)mol/l</th>
<th>Cl = 300 ms</th>
<th>Cl = 500 ms</th>
<th>Cl = 2,000 ms</th>
<th>Transmembrane Potential-Ca Delay, ms</th>
<th>CaT Rise Time, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluo-4FF</td>
<td>9.7</td>
<td>208 ± 34</td>
<td>256 ± 15</td>
<td>287 ± 53</td>
<td>12.4 ± 1.1</td>
<td>31.5 ± 2.8</td>
</tr>
<tr>
<td>Fluo-2LA</td>
<td>6.7</td>
<td>181 ± 55</td>
<td>232 ± 34</td>
<td>277 ± 57</td>
<td>10.8 ± 0.9</td>
<td>27.5 ± 3.4</td>
</tr>
<tr>
<td>Rhod 2</td>
<td>0.57</td>
<td>213 ± 24</td>
<td>279 ± 23*</td>
<td>353 ± 41*</td>
<td>12.0 ± 1.0</td>
<td>28.8 ± 3.8</td>
</tr>
<tr>
<td>Fluo-2MA</td>
<td>0.39</td>
<td>233 ± 8*</td>
<td>321 ± 35*</td>
<td>531 ± 128*</td>
<td>10.1 ± 1.1</td>
<td>24.7 ± 7.1</td>
</tr>
<tr>
<td>Fluo-4</td>
<td>0.35</td>
<td>229 ± 15*</td>
<td>342 ± 47*</td>
<td>458 ± 84*</td>
<td>11.4 ± 1.0</td>
<td>33.0 ± 9.3</td>
</tr>
</tbody>
</table>

Values are means ± SD. CaT, Ca\(^{2+}\) transient; Cl, cycle length. *\(P < 0.05\) vs. Fluo-4FF.
**Data analysis.** Signals were digitally filtered using a low-pass filter with a cutoff frequency of 0.1 kHz to reduce the high-frequency noise. Signals with sufficient signal-to-noise ratio were obtained in ~70% of all recordings. Optical signals were corrected for photobleaching by subtracting a linear signal fit calculated using the baseline signal portion preceding AP upstrokes. The AP and CaT amplitudes were calculated as signal differences before and after the respective upstrokes. The arrival times of APs and CaTs were determined at 50% of signal amplitude. The signal rise times were measured as intervals between moments when signals reached levels of 10% and 90% of the total amplitude. The times to peak were measured as intervals between moments of 10% and 100% of signal values. The durations of APs and CaTs were measured as the time intervals between arrival and recovery times determined at 50% and 80% levels of signal recovery.

Data were expressed as means ± SD. Differences were compared using the two-tailed paired or unpaired *t*-test where appropriate. They were considered significant if *P* < 0.05.

**RESULTS**

Figure 2 shows representative optical recordings of $\text{Ca}^{2+}$ and $V_m$ obtained at different pacing CLs in porcine LV wedge preparation stained by microperfusion at two intramural sites with a high-affinity $\text{Ca}^{2+}$ dye Rhod-2 (site 1) and a low-affinity dye Fluo-4FF (site 2). The Fluo-4FF traces had a lower signal-to-noise ratio than the Rhod-2 traces, likely due to lower affinity of Fluo-4FF. In addition, the preparation was stained by coronary infusion with $V_m$ dye RH-237. The $V_m$ recordings show that APs at the two sites had similar shapes and durations for all CLs. In contrast, CaTs measured with two dyes were different. The CaTs recorded with the low-affinity dye Fluo-4FF (red traces) had durations shorter than CaTs recorded with Rhod-2. At the CL of 300 ms (Fig. 2A), $\text{CaD}_{80}$ measured with Fluo-4FF was ~6% smaller than $\text{CaD}_{80}$ measured with Rhod-2 (181 ms vs. 192 ms, respectively). This difference increased to ~19% (262 ms vs. 322 ms, respectively) when CL was increased to 1,000 ms. Although the CaDs were different, the shapes of CaTs recorded with Rhod-2 and Fluo-4FF dyes were qualitatively similar. In comparison with the local APDs, CaDs measured with Fluo-4FF were somewhat shorter, whereas CaDs measured with Rhod-2 were similar.

Figure 3 compares $\text{Ca}^{2+}$ and $V_m$ recordings obtained in another preparation stained at two sites with low-affinity Fluo-4FF and high-affinity Fluo-4. Similar to Fig. 2, the lower-affinity dye (Fluo-4FF) exhibited lower signal-to-noise ratio than the higher-affinity dye (Rhod-2). Also similar to Fig. 2, APs recorded at two sites had similar shapes and durations at all CLs. At the same time, CaT shapes recorded with Fluo-4FF and Fluo-4 were substantially different, especially at long CLs. At the CL of 300 ms (Fig. 3A), $\text{CaD}_{80}$ measured with Fluo-4 was ~26% longer than that one measured with Fluo-4FF (267 ms vs. 212 ms, respectively). This difference increased to ~114% at a CL of 2,000 ms (557 ms vs. 260 ms, respectively) (Fig. 3D). The CaTs measured with Fluo-4 were significantly longer than the corresponding APs, especially at longer CLs. Thus, at the CL of 2,000 ms, the Fluo-4-$\text{CaD}_{80}$ was ~56% longer than the local APD at 80% level of recovery (557 ms vs. 356 ms, respectively).

In some cases, $\text{Ca}^{2+}$ recordings demonstrated nonmonotonic recovery of CaTs. This is exemplified by the Fluo-4FF recording at CL of 500 ms (Fig. 3B, red trace). This recording shows that the initial $\text{Ca}^{2+}$ recovery was followed by a second rise. In the Fluo-4 recording, this second rise was less apparent but still visible (green trace).

Another interesting feature of $\text{Ca}^{2+}$ recordings is the presence of a long plateau in CaT measured with the Fluo-4 dye at long CLs (Fig. 3D, green trace). This feature could be even more pronounced in other preparations. Figure 4 shows Fluo-4 recordings obtained in another preparation at different CLs in comparison with Fluo-4FF and Rhod-2 recordings. Whereas Fluo-4 CaTs had a typical nearly triangular shape with a rapid rise at CL of 300 ms (lower black trace), the CaT measured at the CL of 500 ms (blue trace) exhibited a long plateau. At the CL of 1,000 and 2,000 ms (lower green and red traces), CaTs exhibited very slow rises with $\text{Ca}^{2+}$ reaching peaks only ~250
ms after the CaT onset. The following Ca\(^{2+}\) recovery was also much slower than at shorter CLs resulting in very long CaTs. Similar results were obtained in a total of 13 preparations. Figure 5 presents data on the duration of CaTs measured using different dyes and corresponding APDs from all preparations. Parameters of Ca\(^{2+}\) transients and corresponding nominal dye \(K_d\) values at selected CLs are also presented in Table 1. In general, increase of dye affinity was associated with prolongation of CaTs. At each CL, the shortest CaTs were measured using the low-affinity dyes Fluo-4FF/Fluo-2LA and the longest CaTs were measured with high-affinity dyes Fluo-4/Fluo-2MA. Rhod-2 measurements exhibited CaTs with intermediate durations. There were no significant differences in average CaDs between the low-affinity dyes Fluo-4FF and Fluo-2LA (NS) or between the high-affinity dyes Fluo-4 and Fluo-2MA (NS). The largest difference in average CaD\(_{50}\) between Fluo-4/Fluo-2MA and Fluo-4FF/Fluo-2LA was ~81\% at CL 2,000 ms (Fig. 5A). The difference between Rhod-2 and Fluo-4FF/Fluo-2LA at this CL was ~35\% (Fig. 5A). The CaD\(_{50}\) measurements exhibited qualitatively similar differences between high- and low-affinity dyes. In contrast with CaD, the APD measurements (Fig. 5, C and D) were not significantly different in all series of measurements.

The nonmonotonic recovery of CaT with the second Ca\(^{2+}\) rise exemplified in the Fluo-4FF trace in Fig. 3B was observed at long CL in 30–50% of recordings obtained with different dyes. The second Ca\(^{2+}\) rise appeared at the beginning of phase 3 of AP with an average delay of 256 ± 42 ms (\(n = 28; CL = 1,000\) ms) after the AP upstroke. The slow rise and signal plateauing shown in Fig. 4 were observed at long CLs in ~50% of recordings with high-affinity Fluo-4/Fluo-2MA. They were not observed in recordings with low-affinity dyes Fluo-4FF/Fluo-2LA, as well as in Rhod-2 recordings. The slow Ca\(^{2+}\) upstroke rise in Fluo-4/Fluo-2MA recordings resulted in prolongation of the time-to-peak of CaTs upon increasing CL (Fig. 6A), which was measured as the time interval between the 10\% level of CaT and its peak. For the low-affinity dyes, this parameter was not significantly changed. There was a trend showing larger CaT time-to-peak values for Fluo-4/Fluo-2MA at long CL in comparison with the low-affinity dyes, but these differences were not statistically significant, probably due to large SD values. There were also no significant changes in the CaT rise times (Fig. 6B) and in the \(V_m\)-Ca delays (Fig. 6C) upon increasing CL for either low-affinity or high-affinity dyes. These parameters also exhibited no significant differences between low- and high-affinity dyes.

**DISCUSSION**

This study investigated the effects of dye affinity on Ca\(^{2+}\) measurements in the whole porcine LV myocardium. The main findings are 1) measurements of Ca\(^{2+}\) transient duration were strongly dependent on the affinity of Ca\(^{2+}\)-sensitive dyes. Dyes with the highest affinity to Ca\(^{2+}\) ions (Fluo-4 or Fluo-2MA) reported significantly longer CaT durations than dyes with the lowest affinity (Fluo-4FF or Fluo-2LA); the dye Rhod-2, which has affinity lower than Fluo-4/Fluo-2MA but higher than Fluo-4FF, exhibited no significant differences between low- and high-affinity dyes.
Fluo-2MA, sensitive dye and optical recordings of tissue structure. Concurrent tissue staining with a series of injections), which could be due to local differences in the diffusion of dye molecules. A disadvantage of the microcapillary staining was that it did not work every time (usable signals were obtained in 70% of injections), which may explain the finding that, despite having a nominal 

It was previously reported that dye affinity could affect Ca$^{2+}$ measurements in cardiac cells. Optical mapping in cultures of neonatal rat myocytes demonstrated that CaD measured with high-affinity calcium dyes were twice as long as CaD measured with low-affinity dyes (5, 6). Whether this is true in whole cardiac tissue was not known. In this work, we compared Ca$^{2+}$ recordings obtained using five Ca$^{2+}$-sensitive dyes with different affinities to calcium ions. The CaD differences between the dyes (Fluo-4, Fluo-2MA, Rhod-2, Fluo-2LA, and Fluo-4FF) were most pronounced at longer CLs reaching ~80% at CL of 2 s. The Rhod-2 dye, which has an intermediate $K_d$ (~0.6 μmol/l), reported moderately prolonged Ca$^{2+}$ transients with the CaD difference from the low-affinity dyes of ~35%. These findings are qualitatively similar to the results obtained in neonatal cell cultures (5, 6).

With the consideration of the $K_d$ values of the dyes, it should be mentioned that these values were obtained in test solutions. Inside cells, the dye $K_d$ may be substantially different. However, the nominal $K_d$ is still useful because dyes that have relatively high affinity to Ca$^{2+}$ ions in a test solution are likely to have a relatively high-affinity inside cells. Therefore, nominal $K_d$ can be used to compare relative dye affinities, especially for dyes with similar molecular structures, such as dyes from the Fluo group. The Rhod-2 dye is different in that regard, which may explain the finding that, despite having a nominal $K_d$ value similar to Fluo-4/Fluo-2MA, the CaD measurements obtained with Rhod-2 were different.

The effect of dye affinity on CaD measurements was most pronounced at long CLs but was less prominent at short CLs. For instance, CaD$_{50}$ values measured with Rhod-2 and Fluo-4FF were not statistically different at CLs of 250 and 300 ms (Fig. 5A and Table 1). This suggests that the role of dye affinity in CaD measurements might be less important in studies of

4FF/Fluo-2LA, resulted in intermediate CaD values. 2) Ca$^{2+}$ transients measured with high-affinity dyes often exhibited signal saturation, whereas saturation was absent in recordings obtained using low-affinity dyes and Rhod-2.

Optical recordings of intracellular calcium demonstrated that the shape of observed Ca$^{2+}$ transients was strongly dependent on the dye affinity. Of the five Ca$^{2+}$-sensitive dyes tested, the dyes Fluo-4 and Fluo-2MA with the highest affinity ($K_d, \sim 0.35 \mu$mol/l) exhibited the longest Ca$^{2+}$ transients in the intact cardiac tissue, whereas the two Ca$^{2+}$-sensitive dyes with the lowest affinity (Fluo-4FF and Fluo-2LA, $K_d = 7$–10 μmol/l) demonstrated the shortest Ca$^{2+}$ transients. The CaD differences between the high- and low-affinity dyes were most pronounced at longer CLs reaching ~80% at CL of 2 s. The Rhod-2 dye, which has an intermediate $K_d$ (~0.6 μmol/l), reported moderately prolonged Ca$^{2+}$ transients with the CaD difference from the low-affinity dyes of ~35%. These findings are qualitatively similar to the results obtained in neonatal cell cultures (5, 6).

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Ca^{2+} TRANSIENTS AND DYE AFFINITY

The role of dye affinity in CaT measurements can be evaluated by considering the linearity of the dye response to changes of Ca^{2+} concentration. This relationship can be presumed linear when Ca^{2+} changes do not exceed the dye $K_d$ value, but it becomes nonlinear at Ca^{2+} concentrations much higher than the dye $K_d$ (19). The Ca^{2+} concentration in cardiac myocytes at the peak of CaT was estimated to be around 0.7–1 mM, which significantly exceeds the $K_d$ of high-affinity dyes. Modeling of dye response to Ca^{2+} changes demonstrated that high-affinity dyes caused changes in CaT shape, which were qualitatively similar to those measured experimentally, whereas low-affinity dyes reproduced CaTs without distortions (6). This qualitative correspondence between the modeling and experimental data supports the explanation that different CaT durations measured in the whole tissue with dyes of different affinities are caused by nonlinear response of the high-affinity dyes. It also indicates that, in the absence of in situ dye calibration, the low-affinity dyes are more appropriate for measurements of Ca^{2+} transients.

An unexpected finding in the present work was the observation of signal plateauing in measurements with high-affinity dyes Fluo-4 and Fluo-2MA. For these two dyes, CaTs frequently displayed long times to reach peak and long recovery times (Fig. 5). The mechanism of this effect is not clear. It cannot be explained by the nonlinear dye response considered above, which assumes that the underlying Ca^{2+} transients are not modified; in such cases, the nonlinear CaT transformation may actually slightly shorten the time to peak (6). A possible explanation of such signal plateauing might be related to strong buffering of calcium ions by the high-affinity dyes and the alteration of the dynamics of Ca^{2+} concentration changes. This question requires further investigation.

Another unexpected finding was the frequent observation of nonmonotonic Ca^{2+} transients with a secondary Ca^{2+} rise occurring during the repolarization phase of AP. A possible explanation of such Ca^{2+} rise could be the motion artifact but this is unlikely for two reasons. First, $V_m$ traces showed little or no distortions at all, although typically optical $V_m$ recordings are more sensitive to the motion artifact than Ca^{2+} measurements (7). Second, these secondary Ca^{2+} deflections were always upward, whereas motion-related deflections can be also downward or biphasic (7). Therefore, it is more likely that these signals reflect the real Ca^{2+} changes. The fact that these secondary Ca^{2+} rises were not paralleled by $V_m$ rises or by large APD changes suggests that they were not caused by electrogenic Ca^{2+} fluxes such as the L-type calcium current (13, 26) or the reverse-mode NCX current (8). Therefore, it is more likely that these Ca^{2+} rises occurred due to spontaneous SR calcium release which per se is not electrogenic and does not directly affect the APD. Although it is known that spontaneous SR calcium release can produce $V_m$ rises in the form of early afterdepolarizations (27), it was also reported that secondary Ca^{2+} rises could occur without accompanying early afterdepolarizations (20), similar to our present observations.

**Summary.** In summary, measurements of calcium transients in porcine ventricular myocardium were strongly affected by the affinity of Ca^{2+} dyes. Overestimation of CaT duration and

arrhythmias with high excitation rates than in Ca measurements during sinus rhythm. In addition, CaD differences between dyes with different affinities might be less pronounced in animals with rapid intrinsic heart rates such as rats and mice.

We have performed similar CaD measurements in several experiments on isolated rabbit hearts paced at a short CL (CL = 300 ms, unpublished data). Although differences in CaD_{80} measured with different dyes were not statistically significant, these measurements demonstrated a trend toward longer CaD with increasing dye affinity.

A question can be asked which dye is more appropriate to measure the shape of Ca^{2+} transients and what is the role of dye affinity in these measurements. Unfortunately, there is no independent method that would allow measuring Ca^{2+} changes with sufficient speed to validate optical measurements. Although Ca^{2+} can be measured with calcium-sensitive microelectrodes (10, 23), their slow response time prevents its application for monitoring fast CaTs in the heart. An alternative method to resolve this problem is to calibrate optical Ca^{2+} measurements in situ following the physiological Ca^{2+} measurements (3). However, as discussed previously (5), such in situ dye calibration in the whole tissue is technically very difficult. Importantly, it requires the use of a ratiometric dye to compensate for signal decline caused by dye photobleaching and washout during the course of experiment. All dyes used in the present study, as well as dyes most often used in the whole tissue studies, are nonratiometric.

Fig. 6. Parameters of CaT upstrokes measured with different dyes as functions of CL. A, CaT time to peak; B, CaT rise time; C, $V_m$-CaD upstream delay.
frequent signal plateauing indicate that high-affinity dyes Fluo-4 and Fluo-2MA misrepresent Ca$_{2+}$ transients in the intact cardiac muscle. The dye Rhod-2 exhibited no signal plateauing and only moderately increased Ca$_{2+}$. Because low-affinity Ca$_{2+}$ dyes could not be used in whole tissue with staining by coronary infusion, Rhod-2 currently appears to be the most suitable dye among the tested dyes for qualitative measurements of Ca$_{2+}$ transients, i.e., measurements of spontaneous calcium release or quantitative measurements of calcium alternans. However, it should be used with caution, especially for quantitatively precise CaD measurements. Ultimately, Ca$_{2+}$ measurements in the whole tissue should use low-affinity dyes, which indicates the need for development of low-affinity dyes with a better loading efficiency.

**Limitations.** Insertion of optical fibers may cause local tissue injury. However, the extent of such injury is likely to be much smaller than in previous measurements of intramura1 $V_m$ and Ca$_{2+}$ changes using wedge preparations. The other limitation is that recordings were made from only few intramura1 sites at the same time and not every local staining resulted in usable analyzed data; W.K. and V.G.F. interpreted results of experiments; W.K. analyzed data; W.K. and V.G.F. interpreted results of experiments; W.K. performed experiments; W.K. and V.G.F. analyzed data; W.K. and V.G.F.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: W.K. performed experiments; W.K. and V.G.F. analyzed data; W.K. and V.G.F. interpreted results of experiments; W.K. prepared figures; W.K. drafted manuscript; W.K. and V.G.F. edited and revised manuscript; W.K. and V.G.F. conceived and designed of research; V.G.F. approved final version of manuscript.

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