Effect of Temperature on Receptor-activated Changes in \([Ca^{2+}]\), and Their Determination Using Fluorescent Probes*

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Several recent studies of intracellular \(Ca^{2+}\) concentration ([\(Ca^{2+}\)]), using fluorescent indicators have involved experiments performed at nonphysiological temperatures, frequently room temperature. In the studies reported here, a reduction in temperature was shown to influence the measurement of [\(Ca^{2+}\)], in two major ways. The first is an effect on the value for the \(K_d\) (apparent dissociation constant) used in the calculation of [\(Ca^{2+}\)]. The \(K_d\) values for indo-1 and fura-2 were found to change with temperature in a manner analogous to the related compounds [ethylenebis(oxyethylenenitrilo)tetraacetic acid and bis(2-amino-5-bromophenoxy)-ethane-\(N,N',N'\)-tetraacetic acid. Experiments showed that the use of previously published \(K_d\) values (measured at 37 °C) in experiments at room temperature results in errors of at least 25% in the calculated [\(Ca^{2+}\)]. The second arises from the differential temperature sensitivities of the physiological processes that make up any particular value of [\(Ca^{2+}\)]. While resting [\(Ca^{2+}\)], was relatively unaffected by reduced temperature (27 °C, 13 °C), changes in [\(Ca^{2+}\)], following stimulation were profoundly influenced and in a way that was not simply predictable. Analysis of the responses indicated that the observed effects resulted largely from a temperature-dependent reduction in the rate of \(Ca^{2+}\) removal from the cytosol together with an additional component, namely a marked reduction in [\(Ca^{2+}\)] entry, at the lower temperature (13 °C). The net result was observed as changes in the initial rise in [\(Ca^{2+}\)] on stimulation, together with more pronounced, effects on the sustained elevation in [\(Ca^{2+}\)], following stimulation. This latter component was markedly increased at 27 °C, but was decreased in cells at 13 °C.

It is clear that changes in [\(Ca^{2+}\)], act as an intracellular signal for a wide variety of cellular responses to a number of different stimuli. The development by Tsien and his coworkers (1, 2) of a series of fluorescent probes that are sensitive to free \(Ca^{2+}\) concentrations and that can be loaded into cells and trapped intracellularly has made possible the measurement of changes in [\(Ca^{2+}\)], in many different cell types. Despite the undoubted benefit that these probes provide, their use in the precise quantification of [\(Ca^{2+}\)], is frequently not without difficulties (see e.g. Refs. 3–6). The most frequent problems that arise are leakage of the dye from the cell, accumulation within intracellular compartments, incomplete intracellular de-esterification of the loaded acetoxyethyl ester with the formation of interfering partial hydrolysis products, and various poorly understood effects of the intracellular milieu on the calibration properties of the dye. Nevertheless, with appropriate controls and precautions, these probes have provided a relatively easy means of evaluating [\(Ca^{2+}\)], in a variety of cell types under different experimental conditions.

Recently, it has become clear that experimenters are increasingly using these probes at reduced temperatures, and very often at room temperature (20–25 °C). The reasons for such use vary, but include attempts to reduce the rate of dye leakage from the cells (see e.g. Refs. 7–9), increased temporal resolution of rapid changes in [\(Ca^{2+}\)], (see e.g. Ref. 10), and/or combining the fluorescence measurements with some other experimental technique where temperature control in the physiological range is difficult or previously not commonly used (e.g. patch clamping; see e.g. Refs. 11 and 12). However, the use of these fluorescent probes at nonphysiological temperatures has several potential implications for the determination of [\(Ca^{2+}\)], most of which do not appear to have been fully incorporated in the studies to date. Most obvious is the fact that, in the calculation of [\(Ca^{2+}\)], the \(K_d\) values (apparent dissociation constants) for the dyes used are generally those originally reported by Grynkiewicz et al. (2) which were obtained at 37 °C. Use of such values with fluorescence measurements made at room temperature, for example, ignores any temperature effect on the \(K_d\) of the dye for \(Ca^{2+}\). Such temperature effects are quite marked in EGTA, the parent compound for these dyes, and also in the related \(Ca^{2+}\) chelators BAPTA and dibromo-BAPTA (13). Clearly, if the \(K_d\) values for the fluorescent probes are similarly affected by temperature the result will be a corresponding systematic error in all calculated values of [\(Ca^{2+}\)]. Furthermore, it is known that [\(Ca^{2+}\)], depends on a variety of separate components of cellular \(Ca^{2+}\) homeostasis and any differential temperature sensitivity among these components could dramatically effect both resting values of [\(Ca^{2+}\)], and the changes that occur on stimulation.

In the studies we report here, we first determined the \(K_d\) values for the two most frequently used \(Ca^{2+}\) probes, indo-1 and fura-2, at temperatures between 37 and 13 °C. The results demonstrate that the temperature dependence of the calcium binding for these dyes is essentially similar to that seen with EGTA and BAPTA, and lead to the conclusion that the inappropriate use of the previously reported \(K_d\) values, measured at 37 °C, in determinations made at lower temperatures will result in significant errors in the estimated values of [\(Ca^{2+}\)], obtained. We then investigated the effect of tem-
perature on resting [Ca\(^{2+}\)], and the changes in [Ca\(^{2+}\)], associated with cell activation in a model exocrine cell, the avian nasal gland. In previous studies (15), we have shown that this cell type is an excellent system for such studies using the fluorescent probes, particularly as it does not show many of the problems of leakage, incomplete de-esterification, etc., referred to above.

**EXPERIMENTAL PROCEDURES**

**Temperature Effects on the \(K_d\) for Indo-1 and Fura-2—**The \(K_d\) values for the fluorescent dyes were measured in standard solutions of the same basic composition as that used by Grynkiewicz et al. (2) as follows: 115 mM KCl, 20 mM NaCl, 1 mM MgCl\(_2\), 10 mM K-MOPS, pH 7.05 at 25°C. The effect of temperature on the \(K_d\) values was determined in the following way. BAPTA (10 mM) was added to the above solution together with different concentrations of CaCl\(_2\) (3.333–7.143 mM) to produce solutions with estimated free [Ca\(^{2+}\)] ranging from approximately 50 to 500 nM. 0.4 μM indo-1 free acid or fura-2 free acid was added and the precise free [Ca\(^{2+}\)] in each of these solutions determined spectrophotometrically at 37°C using the fluorescence ratio technique. All measurements were made using a Perkin-Elmer LS-5B spectrofluorimeter. For indo-1, excitation was at 350 nm and the emission wavelength was alternated between 405 and 485 nm. For fura-2, the excitation wavelength alternated between 340 and 380 nm and emission was at 510 nm. Temperature was regulated by using a thermostatically controlled cuvette holder connected to a circulating water bath. For each solution, background fluorescence was determined by calculating (14). In the standard solutions used, the \(K_d\) for BAPTA at 37°C could be calculated to be 1.15 ± 0.057 × 10\(^{-7}\) M in the indo-1 solutions, and 1.349 ± 0.077 × 10\(^{-7}\) M for the fura-2 solutions.

For each of the experimental temperatures, this \(K_d\) was adjusted according to the equation

\[
pK' = pK + \Delta H(1/T - 1/T')/2.303R
\]

where \(T'\) and \(T\) are the original and experimental temperatures respectively (K), \(pK'\) and \(pK\) are the negative logs of the corresponding \(K_d\) values, \(R\) is the universal gas constant (8.314 × 10\(^{-2}\) kJ/mol), and \(\Delta H\) is the Van't Hoff Isochore for BAPTA, which Harrison and Bers (13) calculated as 13.9 kJ/mol.

Using the appropriately temperature-adjusted \(K_d\) for BAPTA, together with the known total BAPTA and total calcium concentrations, it was possible to calculate the free [Ca\(^{2+}\)] in each of our standard solutions at each temperature (14). The fluorescence ratio of each solution was then determined at the different temperatures, independently, with background fluorescence similarly determined at each temperature and subtracted. From these measured fluorescence ratios, together with the true free [Ca\(^{2+}\)] determined above, it was possible to calculate the appropriate \(K_d\) for the dye for each solution at each temperature.

**Measurement of [Ca\(^{2+}\)] in Isolated Cells—**Intracellular Ca\(^{2+}\) concentrations were determined in isolated cells obtained from the nasal salt-secreting gland of ducklings (Anas platyrhynchos) using the fluorescent probe indo-1 essentially as described previously (15). Resting [Ca\(^{2+}\)], and the changes that occur on activation of muscarinic receptors using the agonist carbachol were determined at three separate temperatures, 38, 27, and 13°C. In each case, the cell suspensions were preincubated to the relevant temperature for a minimum of 30 min and temperature was maintained during the measurements using the thermostatically controlled cuvette holder of the spectrofluorimeter.

**RESULTS**

The effect of temperature on the \(K_d\) for indo-1 and fura-2 is illustrated in Fig. 1 which shows the mean ± S.E. (n = 4) of the \(K_d\) values determined in each of the standard solutions at the different temperatures (Fig. 1A), and an Arrhenius plot of the same data (Fig. 1B). The lines represented in the latter were obtained by linear regression analysis of the data, and their slopes are equal to \(-\Delta H/2.303R\) (13). From this it can be calculated that the \(\Delta H\) of the \(K_d\) for indo-1 is 12.5 kJ/mol, and for fura-2 is 10.3 kJ/mol. These values compare with those reported by Harrison and Bers (13) for EGTA (165 kJ/mol), BAPTA (13.9 kJ/mol), and dibromo-BAPTA (16.9 kJ/mol), and indicate that the \(K_d\) for indo-1 and for fura-2 shows a similar sensitivity to temperature. As suggested above, such a marked temperature dependence can be expected to result in significant errors in the calculated values of free [Ca\(^{2+}\)] if the inappropriate \(K_d\) is used. As an illustration of this, it can be shown that the use of the previously reported \(K_d\) values (measured at 37°C) in determinations of free [Ca\(^{2+}\)] made at room temperature (22°C) would result in underestimations of 27 and 25% for indo-1 and fura-2, respectively.

Measurements of [Ca\(^{2+}\)] in isolated cells of the avian nasal gland at three different temperatures (38, 27, and 13°C) using the corrected \(K_d\) showed that resting [Ca\(^{2+}\)] was only slightly affected by a reduction of temperature. However, following activation of muscarinic receptors using carbachol (500 μM)

![Fig. 1. Effect of temperature on the apparent dissociation constant \((K_d)\) for Ca\(^{2+}\) of fura-2 and indo-1. A, the \(K_d\) values (nanomolar) for fura-2 (1) and indo-1 (2) were determined at different temperatures between 13 and 37°C as described under "Experimental Procedures." Each point represents the mean ± S.E. of determinations made at four different free Ca\(^{2+}\) concentrations between 50 nM and 500 nM. B, an Arrhenius plot of the same data where the negative log of the \(K_d\) (pKD) is plotted against the reciprocal of the temperature (K). Lines were obtained by linear regression analysis of the data, and their slopes are equal to \(-\Delta H/2.303R\) (see text).](image-url)
Temperature Effects on Fluorescence Measurements of \([\text{Ca}^{2+}]_i\)

The changes in \([\text{Ca}^{2+}]_i\) were dramatically changed by reducing the temperature (Fig. 2). At 38°C, the normal response of a rapid rise in \([\text{Ca}^{2+}]_i\), followed by a slower decline to a sustained plateau of around 350 nM was seen. This plateau was maintained for as long as receptor activation was continued and on addition of the antagonist atropine (100 μM) \([\text{Ca}^{2+}]_i\) fell rapidly to a level not significantly different from resting levels. At 27°C, an essentially similar rapid rise in \([\text{Ca}^{2+}]_i\) was seen but this was not followed by any significant decline and, instead, \([\text{Ca}^{2+}]_i\) remained elevated at a sustained value around 460 nM, some 40% higher than that seen at 38°C. As before, addition of atropine rapidly returned \([\text{Ca}^{2+}]_i\) to resting levels. At 13°C, addition of carbachol sharply increased \([\text{Ca}^{2+}]_i\), but at a rate clearly slower than that seen at either 38 or 27°C. Again, this was followed by a gradual decline before finally stabilizing at a sustained plateau value of around 250 nM, approximately 25% lower than that seen at 38°C. Addition of atropine again rapidly restored resting levels of \([\text{Ca}^{2+}]_i\).

The above data indicated that temperature was having profound, and complex, effects particularly on the changes in \([\text{Ca}^{2+}]_i\), seen following receptor activation. In order to analyze these effects more precisely, an attempt was made to study the \([\text{Ca}^{2+}]_i\) mobilization and \([\text{Ca}^{2+}]_i\) entry components of the overall \([\text{Ca}^{2+}]_i\) signal separately. To do this we employed the protocol of initially stimulating the cells while in a low-\([\text{Ca}^{2+}]_i\) medium (40 μM) to reveal the \([\text{Ca}^{2+}]_i\) mobilization phase, followed by the subsequent restoration of normal \([\text{Ca}^{2+}]_i\) (1.3 mM) to reveal the receptor-activated \([\text{Ca}^{2+}]_i\) entry component. As discussed previously (15), we have determined that suspension of the cells in media with a \([\text{Ca}^{2+}]_i\) of 40 μM does not significantly deplete intracellular \([\text{Ca}^{2+}]_i\) stores or affect resting \([\text{Ca}^{2+}]_i\), and membrane \([\text{Ca}^{2+}]_i\) permeability, yet minimizes any sustained elevation in \([\text{Ca}^{2+}]_i\), following receptor activation suggesting that receptor-activated \([\text{Ca}^{2+}]_i\) entry is minimal under such conditions. Fig. 3 shows that activation of muscarinic receptors in cells suspended in the low-\([\text{Ca}^{2+}]_i\) medium produces similarly rapid increases in \([\text{Ca}^{2+}]_i\), at the three temperatures. However, the overall magnitude of the observed rise is significantly increased at the lower temperatures, being approximately 18% larger at 27°C and 46% larger at 13°C. In addition, there was some indication of an increased delay in the onset of this rise in \([\text{Ca}^{2+}]_i\), at 13°C. However, exact quantification of this observed delay and its dependence on

**DISCUSSION**

It is clear from the above that, as might have been predicted from the nature and origins of the molecules involved, temperature has significant effects on the \(K_v\) values of both indo-1 and fura-2. It should be noted that in the protocol used in these measurements the fluorescent probes were used under

![Fig. 3. Effects of \([\text{Ca}^{2+}]_i\), on changes in \([\text{Ca}^{2+}]_i\), following muscarinic receptor activation at different temperatures. \([\text{Ca}^{2+}]_i\), was determined in isolated cells loaded with indo-1 and suspended in low-\([\text{Ca}^{2+}]_i\) medium ([\(\text{Ca}^{2+}]_i\), = 40 μM) at different temperatures. At the point indicated (A) 500 μM carbachol was added, followed 4 min later by sufficient extracellular \(\text{CaCl}_2\) to raise \([\text{Ca}^{2+}]_i\), to 1.3 mM (B). Atropine (100 μM) was finally added 4 min later (C). Results obtained from cells at 27 and 13°C are compared with cells at 38°C (broken line). Each trace represents the mean results from nine separate experiments performed on three different cell isolates at each temperature. The S.E. of these traces never exceeded 10% of the mean and was more usually less than 5% of the mean value.

![Fig. 2. Changes in \([\text{Ca}^{2+}]_i\), in isolated cells at different temperatures following muscarinic receptor activation. Cells were isolated and loaded with indo-1 as described under "Experimental Procedures." After incubation at the appropriate temperature, indo-1 fluorescence was measured and \([\text{Ca}^{2+}]_i\), calculated as described. At the point indicated (A) 500 μM carbachol was added, followed 4 min later by 100 μM atropine (B). Representative traces from cells maintained at 27 and 13°C are compared with cells at 38°C (broken line).](image-url)
The experiments measuring \([Ca^{2+}]_i\) in isolated cells and the changes that occur on stimulation further reveal that temperature, in addition to changing the \(K_d\) for the fluorescent probe used, has profound and complex effects on the nature of the response observed. As such, temperature appears to influence both the absolute magnitude of the \([Ca^{2+}]_i\) changes observed, and also the time course of the different components of the response. The complex nature of the observed effects is presumably due to the fact that, at any one time, \([Ca^{2+}]_i\), reflects a balance between an array of diverse processes (e.g. conductive fluxes via channels, carrier-mediated transport systems, buffering etc.) all of which might be expected to demonstrate individual temperature sensitivities.

Despite these undoubted complexities, certain features of the effects of temperature on this system are apparent from the experiments reported here. Thus, in the low-Ca\(^{2+}\) medium, the initial rise in \([Ca^{2+}]_i\), following stimulation largely reflects the Ins(1,4,5)P\(_3\)-induced release of Ca\(^{2+}\) from intracellular stores following receptor activation. The data in Fig. 3 indicate that the overall rate of this agonist-induced release of Ca\(^{2+}\) is not significantly affected by temperature over the range used. Several more direct studies have also found that the Ins(1,4,5)P\(_3\)-induced release of intracellular Ca\(^{2+}\) is relatively temperature-insensitive (17–20).

After the initial peak in \([Ca^{2+}]_i\), the subsequent decline is largely due to a combination of the uptake of Ca\(^{2+}\) into Ins(1,4,5)P\(_3\)-insensitive stores and the pumping out of cytosolic Ca\(^{2+}\) into the extracellular medium. Figs. 3 and 4 show that the overall rate of these processes for the removal of Ca\(^{2+}\), are significantly reduced at 27 °C and further reduced at 13 °C. Such a reduction in the rate of Ca\(^{2+}\) removal at low temperatures would also account for the increased magnitude of the overall change in \([Ca^{2+}]_i\), following initial stimulation (i.e. in the face of a reduced rate of Ca\(^{2+}\) removal, release of a fixed amount of Ca\(^{2+}\) from agonist-sensitive stores will transiently raise \([Ca^{2+}]_i\), to a greater extent at the lower temperatures). Conversely, the increase in \([Ca^{2+}]_i\), seen on restoration of normal \([Ca^{2+}]_i\), largely reflects the rate of Ca\(^{2+}\) entry from the extracellular medium (15). Examination of Fig. 3 shows that this is relatively unaffected by reduction of the temperature to 27 °C but, at 13 °C, the very slow rise in \([Ca^{2+}]_i\), clearly indicates a greatly reduced rate of Ca\(^{2+}\) entry. The conclusion is that lowering experimental temperature to 27 °C slows the rate of Ca\(^{2+}\) removal from the cytosol. This effect is more pronounced at 13 °C but, in addition, there is a profound reduction in the rate of agonist-induced Ca\(^{2+}\) entry which is not seen at 27 °C.

Physiologically, the most important feature of the overall Ca\(^{2+}\) signal affected by temperature is the sustained plateau of elevated \([Ca^{2+}]_i\), following stimulation, as this has been specifically shown to be the critical component in the cholinergic activation of ion secretion (15). From the above analysis of the data obtained, it is possible to explain the observed effects of temperature on this parameter. Thus, at 27 °C, the rate of Ca\(^{2+}\) removal from the cytosol is significantly reduced while the rate of Ca\(^{2+}\) entry following receptor activation is largely unaffected. Assuming that the overall rate of Ca\(^{2+}\) removal from the cytosol is dependent on the level of \([Ca^{2+}]_i\), it can be predicted that the system will come into equilibrium (i.e. removal = entry) at a higher value of \([Ca^{2+}]_i\), thereby explaining the significantly elevated plateau concentration seen at 27 °C. At 13 °C the rate of Ca\(^{2+}\) removal is further slowed but, in addition, receptor-activated Ca\(^{2+}\) entry is also significantly reduced. If it is assumed that the decline in Ca\(^{2+}\) entry exceeds the reduction in Ca\(^{2+}\) removal, then the sus-

**Fig. 4. Temperature dependence of the rate of \([Ca^{2+}]_i\) removal.** The decline in \([Ca^{2+}]_i\), following the transient initial rise in \([Ca^{2+}]_i\), seen on addition of carbachol was obtained for each of the means recorded in Fig. 3. Those values that fell within a common overlapping range of \([Ca^{2+}]_i\), (between 307 and 115 nm) were plotted as log\(_{10}\)\(\left(\frac{[Ca_0] - [Ca_{ul}]}{[Ca_0] - [Ca_{il}]}\right)\) versus time.

- Conditions in which their respective \(K_d\) values were known (2) in order to determine the original free \([Ca^{2+}]_i\) in the standard solutions. This was then used to obtain the \(K_d\) for BAPTA in our original solutions. An alternative procedure would have been to use previously published values for the \(K_d\) of BAPTA (13), apply appropriate corrections to this value for the particular conditions of ionic strength, temperature, and pH in our original standard solutions, and use this to determine the \(K_d\) values for the fluorescent probes in our solutions. The disadvantages of this latter approach are that it is subject to uncertainties as to the precise value of the initial \(K_d\) and the appropriate correction factors, as well as the purity of the BAPTA used.

- It should also be pointed out that no attempt was made to correct the pH of the standard solutions (7.05 at 25 °C) for temperature, BAPTA was specifically chosen as the Ca\(^{2+}\) buffer for the standard solutions to minimize the effects of any pH changes on the buffering system. However, it is possible that temperature-induced changes in pH could have affected the \(K_d\) values obtained for indo-1 and fura-2. Preliminary experiments indicated that pH in our solutions increased from 6.93 at 37 °C to 7.15 at 13 °C. Grymkiewicz et al. (2) failed to detect any significant effect of pH within the physiological range (between 7.05 and 6.75) on the \(K_d\) for fura-2. Gunter et al. (16) have shown that the \(K_d\) for either indo-1 or fura-2 is relatively insensitive to increases in pH but is significantly affected by decreases in pH, with indo-1 being more sensitive than fura-2. Examination of their unpublished data indicates that the pH changes in our solutions may have affected the measured \(K_d\) values by approximately 5% at most. More importantly, as pH increased at the lower temperatures, the observed increase in \(K_d\) with reduced temperature would, if anything, be underestimated.

- The demonstration of 25% or greater errors in the calculated values of \([Ca^{2+}]_i\), when \(K_d\) values measured at 37 °C are used in determinations performed at room temperature illustrate the importance of making the appropriate temperature correction for the value of \(K_d\) used. The values determined here for the \(\Delta H\) for the \(K_d\) values of indo-1 and fura-2 permit the calculation of the true \(K_d\) at any temperature between 37 and 13 °C, by simple substitution in Equation 1 given above, and allow the appropriate corrections to be performed for experimental temperatures within this range.
tained plateau value of $[\text{Ca}^{2+}]$, following stimulation will be reduced, as was observed.

One additional feature of the observed temperature effects on the $\text{Ca}^{2+}$ signal is worthy of mention. Examination of the initial increases in $[\text{Ca}^{2+}]$, on addition of carbachol show that reduction of the temperature to 13 °C results in a significant slowing of the rate of rise in $[\text{Ca}^{2+}]$, in those experiments performed at normal $[\text{Ca}^{2+}]$, (Fig. 2), while no such difference is observed in the low-$\text{Ca}^{2+}$ medium (Fig. 3). It has been suggested above that the Ins(1,4,5)P$_3$-induced $\text{Ca}^{2+}$ release is largely unaffected by temperature, while $\text{Ca}^{2+}$ entry is significantly reduced at 13 °C. It therefore seems likely that the observed effects on the rise in $[\text{Ca}^{2+}]$, reflect the fact that in low-$\text{Ca}^{2+}$ media this initial increase is due solely to the release of $\text{Ca}^{2+}$ from agonist-sensitive stores and, as such, is essentially temperature insensitive, while at normal $[\text{Ca}^{2+}]$, the rise in $[\text{Ca}^{2+}]$, via Ins(1,4,5)P$_3$-induced release is supplemented by a component of receptor-activated $\text{Ca}^{2+}$ entry which is significantly reduced at 13 °C. Thus, exactly as observed, the rate of increase in $[\text{Ca}^{2+}]$, would be expected to be unaffected by temperature when measured in low-$\text{Ca}^{2+}$ medium, but would be significantly slowed at 13 °C when measured at normal $[\text{Ca}^{2+}]$. The fact that the temperature-sensitive component of the increase in $[\text{Ca}^{2+}]$, appears to be additive to the Ins(1,4,5)P$_3$-induced release, lends support to the idea previously proposed that receptor-activated $\text{Ca}^{2+}$ entry occurs via a separate pathway operating in parallel with, and in addition to, the release of $\text{Ca}^{2+}$ from agonist-sensitive intracellular stores (21).

In conclusion, a reduction in experimental temperature has multiple implications for the observed changes in $[\text{Ca}^{2+}]$, induced on cell activation and in the use of fluorescent $\text{Ca}^{2+}$ probes for their determination. The effects on the properties of the fluorescent probes are essentially fairly easy to predict and can be correct by appropriate means. The effects on the actual changes in $[\text{Ca}^{2+}]$, are more complex and difficult to predict, and involve the distinct differential temperature sensitivities of the separate components that contribute to the overall response.

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