Plasmalemmal sodium-calcium exchanger shapes the calcium and exocytotic signals of chromaffin cells at physiological temperature

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Padín JF, Fernández-Morales JC, Olivares R, Vestring S, Arranz-Tagarro JA, Calvo-Gallardo E, de Pascual R, Gandía L, García AG. Plasmalemmal sodium-calcium exchanger shapes the calcium and exocytotic signals of chromaffin cells at physiological temperature. Am J Physiol Cell Physiol 305: C160–C172, 2013.—The activity of the plasmalemmal Na+/Ca2+ exchanger (NCX) is highly sensitive to temperature. We took advantage of this fact to explore here the effects of the NCX blocker KB-R7943 (KBR) at 22 and 37°C on the kinetics of Ca2+ currents (Ic), cytosolic Ca2+ ([Ca2+]i) transients, and catecholamine release from bovine chromaffin cells (BCCs) stimulated with high K+ (30–80 mM), caffeine, or histamine. At 22°C, the effects of KBR on those parameters were meager or nil. However, when the electrochemical gradient for Na+ was reversed such as during membrane depolarization or the opening of Na+ channels, the exchanger moves Na+ out of the cell and Ca2+ into the cell (3, 6). The Ca2+ exit mode is referred to as the forward mode (fNCX) and the Ca2+ entry mode as the reverse mode (rNCX). The plasmalemmal NCX has three isoforms, NCX1, NCX2, and NCX3, encoded by separate genes, and their distribution is tissue specific (20). Bovine chromaffin cells (BCCs) express the major isoform NCX1 (29, 31), which can mediate Na+-dependent Ca2+ influx (22) or Ca2+ export (33), depending on the experimental conditions.

On the basis of experiments performed with ouabain to augment the cytosolic Na+ concentration (11, 14) or the manipulation of external Na+ and Ca2+ concentrations (31) that elicited enhanced catecholamine release responses, the proposition was made that the NCX could intervene in the long-term regulation of such exocytotic responses in BCCs (9, 21, 22, 30, 31, 35).

Those experiments were performed at room temperature whereby the NCX contributes to the clearance of depolarization-elicited cytosolic Ca2+ ([Ca2+]i) elevations at a maximal rate of 20 μmol·l cells−1·s−1 in rat chromaffin cells (32). However, in K+ stimulated BCCs at 37°C the rate of Ca2+ eflux is close to 100 μmol·l cells−1·s−1 (36), fivefold higher. It therefore follows that any cell function depending on the generation of a [Ca2+]i transient and its subsequent clearance by the NCX contribution will be best unmasked at 37°C.

In this study we took advantage of the strong temperature dependence of the NCX to explore its contribution to shaping the [Ca2+]i transients and the exocytotic catecholamine release responses in BCCs challenged with short K+ depolarizing pulses (DPs) at 22 and 37°C. To block the NCX we recourse KB-R7943 (KBR), an iso thiourea derivative that blocks the cardiac NCX at 1–5 μM (39). In some experiments we also used the compound SEA0400 (SEA), a more potent and selective NCX inhibitor in cultured neurons (25). We found that at the physiological temperature of 37°C the NCX plays a much more significant role in regulating the shapes of [Ca2+]i transients and exocytotic responses than previously thought with experiments performed at room temperature.

METHODS

Ethical approval. Bovine adrenal glands were obtained from a local slaughterhouse that legally and ethically killed the calves for human feeding. Thus in this case the ethical issues on the use of animals for laboratory experiments, in accordance with the Declaration of Helsinki and the approval of the Ethical Committee for the Care and Use of Animals of the Medical School, Autonomous University of Madrid, Spain, in accordance with the Directive 2010/63/EU of the European
700-mV potential was applied to the electrode with respect to an AgCl electrode. The electrodes were calibrated according to good amperometric practices (24) by perfusing 50 μM norepinephrine dissolved in standard Tyrode solution and measuring the current elicited; only electrodes that yielded a current between 200 and 400 pA were used. The coverslips were mounted in a chamber on a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan) used to localize the target cell, which was continuously superfused by means of an eight-way superfusion system with a common outlet driven by electrically controlled valves. To achieve this, the Tyrode solution was composed as follows (in mM): 137 NaCl, 1 MgCl2, 5.3 KCl, 2 CaCl2, 10 HEPES, and 10 glucose, and 10 HEPE, pH 7.3 with NaOH, 22 or 37°C. The high K+ solution was prepared by replacing equisomolar concentrations of NaCl with KCl. At the time of experiment performance, proper amounts of drug stock solutions were freshly dissolved into the Tyrode solution.

Online amperometric recordings of burst catecholamine release from superfused populations of BCCs. Cells were scrapped off carefully from the bottom of the Petri dish (5 × 10^6 cells per dish) with a rubber policeman and centrifuged at 120 × g for 10 min. The cell pellet was resuspended in 200 μl of Krebs-HEPES (composition in mM: 144 NaCl, 5.9 KCl, 1.2 MgCl2, 2 CaCl2, 11 glucose, and 10 HEPES, pH 7.3 with NaOH). Cells were introduced in a 100-μl microchamber for their superfusion at the rate of 2 ml/min. The liquid flowing from the superfusion chamber reached an electrochemical detector model VA 641 (Metrohm, Herisau, Switzerland) placed just at the outlet of the microchamber, which monitors online the amount of catecholamines secreted under the amperometric mode. Cells were stimulated to secrete with short pulses (5 s) of a Krebs-HEPES solution containing 75 mM K+ (75K+) with isosmotic reduction of Na+. Solutions were rapidly exchanged through electrowaves commanded by a computer. This amperometric strategy permits the online recording of reproducible catecholamine release responses during long time periods of 30–60 min.

Chemical products. Products to make saline solutions and compounds FPL 64176 (FPL), caffeine, and histamine were purchased from Sigma (Sigma-Aldrich, Madrid, Spain). KBR was obtained from TOCRIS Bioscience (Bristol, UK) and SEA was from Shanghai TOCRIS Bioscience (Bristol, UK). L-glutamine was from Sigma (Sigma-Aldrich), fetal bovine serum was from PAA Laboratories (Pasching, Austria), and collagenase-A from Clostridium histolyticum was from Roche Diagnostics (Mannheim, Germany). The probe fura-2 AM was supplied by Invitrogen (Eugene, OR).

Data analysis and statistics. Whole-cell inward Ica amplitude was measured at the maximum peak current during the 50-ms DP. Regarding Ica, data analysis was carried out on a personal computer, and data obtained by PULSE software (HEKA-Elektronik) and IgorPro (Wavemetrics, Lake Oswego, OR) were exported to Excel tables (Microsoft, Redmond, WA). Only the cells that held up the entire protocol (control, 3 μM KBr, and wash) were included in the statistics. We used a Student’s t-test for comparison between control or wash and drug current-voltage (I-V) curves at each depolarizing voltage step. Data are represented as means ± SE of at least two different cultures. Kinetic parameters of [Ca2+]c, transients and Ica time constants (τon, τ of activation; τinact, τ of inactivation; and τdeact, τ of deactivation) were determined using Origin Pro 8 SR2 software, version 8.0891 (OriginLab, Northampton, MA). The [Ca2+]c transients and calcium currents records were fitted to the equation

\[ y = \text{plateau} + (y_0 - \text{plateau}) \cdot e^{-x/t} \]  

where \( x \) is the time, \( y \) starts at \( y_0 \) and goes down or up to a plateau, \( t \) is the time or rate constant equal to the reciprocal of the \( x \)-axis. Regarding [Ca2+]c, data analysis was carried out on a personal computer, and data obtained from LAS AF Lite software, version 2.6.0 (Barcelona, Spain) were exported to Excel tables. Graphs and the mathematical analyses were performed using the Graphpad Prism software, version 5.01 (GraphPad Software, San Diego, CA). Areas or
peaks heights were calculated by integrating the calcium transient over time during the stimulus duration by means of Origin Pro 8 SR2 software, version 8.0891. Results shown in the text and figures are expressed as means ± SE. Unless otherwise stated, statistical analyses was carried out with ANOVA one-way test, and Tukey post hoc analyses (*P < 0.05, **P < 0.01, or ***P < 0.001, statistical signification).

For amperometric recordings data analysis was carried out on a personal computer using Excel and IgorPro. Amperometric charge (Qamp) was calculated by integrating the amperometric current over time during the stimulus duration with a macro written in IgorPro. The number of spikes >7 pA was manually counted on an extended graph displayed in the computer screen. A ruler was drawn at 7 pA, and spikes going above the threshold amplitude were considered. Differences between means of group data fitting a normal distribution were assessed by using either ANOVA or Kruskal-Wallis test for comparison among multiple groups or Student’s t-test for comparison between two groups; P < 0.05 was taken as the limit of significance. Only the cells that responded with >15 spike events during an initial 10-s high K+ DP and that held up the entire protocol (control, drug, and wash) were included in the statistics. Data are represented as means ± SE of at least 3 different cultures (*P < 0.05, **P < 0.01 with respect to control, Mann-Whitney’s rank-sum test; P < 0.05 was taken as the limit of significance).

Single spike analysis was carried out by means of an Igor macro available from Sulzer Laboratory (Columbia University, NY; written by E. Mosharov, http://www.sulzerlab.org/download.html). Recordings were filtered offline at 1 kHz. The mean of all spikes for every parameter was calculated cell by cell. Tests for statistical differences between groups were performed using these means, i.e., one value per cell and per parameter, which is a more robust means of statistical testing (8). Means ± SE are indicated throughout the text (see also Table 3). Data sets were tested for normality and when groups of data were not normally distributed, nonparametric tests were used. Statistical analysis was performed using SigmaPlot and Excel.

RESULTS

The effects of KBR on I_Ca and on the kinetics of the K^+ elicited [Ca^{2+}]_c elevations and catecholamine release responses were explored at 22 and 37°C. Being an ion transporter, NCX has been shown to work more slowly at the lower compared with the higher physiological temperature in chromaffin cells (32, 36). Therefore, the effects of its blockade with KBR should differ at 37°C compared with 22°C. In most experiments the concentration of 3 μM KBR was used; in few experiments, 1 and 10 μM KBR were also used. At these concentrations, the compound is known to efficiently block the NCX of cardiac cells (39).

Effects of temperature and KBR on I_Ca. We have previously shown that a 100-ms DP applied to a voltage-clamped BBC generates an inward I_Ca and a [Ca^{2+}]_c transient nearby sub-plasmalemmal sites; few seconds thereafter the transient propagates through inner cytosolic sites as a Ca^{2+} wave (1). As in the present study we wanted to find out that if the NCX was involved in the regulation of [Ca^{2+}]_c transients and exocytotic signals, then it was of paramount importance to know if temperature and KBR affected I_Ca.

Cells were voltage clamped at −80 mV under the perforated patch configuration of the patch-clamp technique using 2 mM Ca^{2+} as the charge carrier. Then, they were regularly challenged with 50-ms DPs given in a step-wise manner every 15 s and at 10-mV steps. The application of three subsequent protocols given to the same cell produced highly reproducible I–V curves. Therefore, 3 μM KBR was given 2 min before and during the second I–V protocol; the first was taken as control and the third as recovery after KBR washout. A family of I_Ca traces obtained at different voltages in an example cell perfused at 22°C is shown in Fig. 1A. I–V curve pooled data from 13 cells at 22°C are shown

![Fig. 1. Effects of temperature and KB-R7943 (KBR) on inward Ca^{2+} currents (I_Ca). Recording of I_Ca was done under the perforated patch configuration of the patch-clamp technique. Cells were voltage-clamped at −80 mV and continuously perfused with and extracellular solution containing 2 mM Ca^{2+}. At 15-s intervals, cells were stimulated with 50-ms depolarizing pulses (DPS) given at 10-mV steps to the voltages (V_m) indicated in the current-voltage (I–V) curves of B and D. Three sequential I–V curves (control, in the presence of 3 μM KBR given since 2 min before, and during the entire period of the I–V curve and wash), at 22°C (A and B) and 37°C (C and D). Families of I_Ca traces at different voltages of an example cell are shown in A (22°C) and C (37°C). I–V curves were obtained by monitoring I_Ca peak amplitude at each voltage; data points are means ± SE of the number of cells and cultures shown in parentheses.](http://ajpcell.physiology.org/)
in Fig. 1B. Threshold $I_{Ca}$ was at −40 mV, $I_{Ca}$ peaked at −20/−10 mV, and the reversal potential was at 60 mV. Current kinetic parameters are displayed in Table 1.

A family of $I_{Ca}$ traces at the different voltages tested from an example cell perifused at 37°C is shown in Fig. 1C. I-V curve pooled data from 10 cells at 37°C are shown in Fig. 1D. Threshold $I_{Ca}$ was at −40 mV, $I_{Ca}$ peaked at −10 mV, and the reversal potential was at 60 mV. $I_{Ca}$ kinetic parameters are shown in Table 1. $I_{Ca}$ had a 1.7-fold higher amplitude at 37°C, compared with 22°C. Current activation was 2.6-fold faster and deactivation was 2-fold slower at 37°C, compared with 22°C. Hence, total Ca$^{2+}$ entry during a 50-ms DP in 2 mM extracellular [Ca$^{2+}$] ($Q_{Ca}$) was 48% higher at 37°C, compared with 22°C (Table 1). The I-V curve in the presence of KBR overlapped with the control I-V curve at both 22°C (Fig. 1B) and 37°C (Fig. 1D). The family of $I_{Ca}$ traces at different voltages obtained in the presence of KBR was also quite similar to control and wash traces at both temperatures (Fig. 1, B and D). This lack of effect of KBR on the kinetic parameters of $I_{Ca}$ at 22 and 37°C is better seen in Table 1; the compound did not significantly modify $\tau_{a}$, $\tau_{r}$, $\tau_{d}$, and $Q_{Ca}$ at both temperatures indicating that at 3 μM KBR did not bind to voltage-dependent Ca$^{2+}$ channels (VDCCs) in the present experimental conditions.

Effects of temperature and KBR on the kinetics of K$^{+}$-elicited [Ca$^{2+}$], transients. We explored next how temperature and KBR affected the kinetics of [Ca$^{2+}$]$_{i}$ transients elicited by 10-s K$^{+}$ DPs, applied onto fura-2-loaded BCCs. The stimulating solution contained 45 mM K$^{+}$ (isosmotic reduction of Na$^{+}$) and 2 mM Ca$^{2+}$; it is known that at this concentration K$^{+}$ shifts the membrane potential ($E_{m}$) to −25 mV in current-clamped BCCs (28), a cell depolarization that causes near maximum peak $I_{Ca}$ at 22 and 37°C (Fig. 1, B and D). The protocol used here consisted in the application of seven DPs to the same cell (P1 to P7) at 2-min intervals. The amplitude and shape of the [Ca$^{2+}$]$_{i}$ transients were quite similar in control cells; cells were discarded when the peak amplitude of the transients decayed >20%.

Figure 2A shows an example cell stimulated with K$^{+}$ pulses at 22°C. The [Ca$^{2+}$]$_{i}$ transients were quite similar before (P1 to P3) and during KBR treatment (P4 to P6); only P6 and P7 (KBR washout) had smaller amplitudes. The kinetic parameters of the [Ca$^{2+}$]$_{i}$ transients generated by pulses P3 (control) and P4 (in the presence of KBR) are given in Table 2; amplitude, width, and area were similar indicating that the total Ca$^{2+}$ entry was unaffected by KBR. Furthermore, decay parameters indicating [Ca$^{2+}$]$_{i}$ clearance such as elimination constant ($k_{e}$), half-time ($t_{1/2}$), and time constant ($\tau$) were not significantly modified by KBR. These parameters allowed the representation of the mean P3 and P4 transients from the pooled results of 14 cells from 3 different cultures (Fig. 2B); both spike transients practically overlapped.

At 37°C, the transient amplitudes were quite reproducible as indicated by records taken from an example cell (Fig. 2C). In the presence of KBR, a decrease of amplitude and a slower decay were observed (Fig. 2C, dotted rectangle). Most kinetic parameters showed a statistical difference between P3 (control) and P4 (KBR) (Table 2). Thus KBR decreased the amplitude of the transient by 25.8%, area by 28.9%, and $k_{e}$ by 47.5%; in concordance with this smaller rate of clearance $t_{1/2}$ rose by 90.7% and $\tau$ by 91.4%. Plots of the [Ca$^{2+}$]$_{i}$ transients drawn with pooled kinetic parameters averaged from 12 cells showed a smaller amplitude; the rate of clearance was considerably slowed down in the presence of KBR (Fig. 2D). We also made statistical comparisons between the kinetic parameters of control (P3) transients at 22 and 37°C (Table 2). At 37°C, $k_{e}$ was 24.5% smaller, while $t_{1/2}$ and $\tau$ were 40.6 and 38.6% higher with respect to 22°C. The superimposed control P3 [Ca$^{2+}$]$_{i}$ transients at 22 and 37°C indicated a quite similar profile (Fig. 2E). In contrast, in the presence of KBR the superimposed [Ca$^{2+}$]$_{i}$ transients at 37°C had a smaller amplitude and a drastic slower clearance, compared with 22°C (Fig. 2F).

Effects of temperature and KBR on the K$^{+}$-elicited quantal release of catecholamine. Modification of the kinetics of [Ca$^{2+}$]$_{i}$ transients by temperature and KBR upon cell depolarization with K$^{+}$ was expected to also change the extent and kinetics of the quantal release of catecholamine. This was explored with experimental protocols similar to those previously used for [Ca$^{2+}$]$_{i}$ transients. Thus, after carbon fiber positioning, the selected BCC was challenged with three successive 10-s DPs (P1, P2, and P3) given at 5-min intervals with a solution containing 30 mM K$^{+}$ (isosmotic reduction of Na$^{+}$ and 2 mM Ca$^{2+}$, the 30K$^{+}$ solution). It is known that at this concentration K$^{+}$ shifts the $E_{m}$ of current-clamped BCCs to −30 mV (28); at this $E_{m}$, $I_{Ca}$ was 50% of peak $I_{Ca}$ (Fig. 1, B and D) indicating a substantial opening of VDCCs.

Challenging the cell with 30K$^{+}$ elicited a burst of secretory spikes that were more frequent and larger at 37°C compared with 22°C, as shown in the P1 pulse of example cells of Fig. 3, A and B. Spike firing began soon after the 30K$^{+}$ application, were visible along the pulse, and frequently overlasted by few seconds the duration of the pulse. Of note was baseline elevation of the initial spike burst during P1 at 37°C, an indication of overlapping spike events that were simultaneously occurring (Fig. 3B). As previously observed (12) in the present study, we also found that BCCs rarely exhibited spontaneous single exocytotic events; this made easier the analysis of the net responses elicited by 30K$^{+}$.

Thus the net bulk secretion (the cumulative sum of the areas of all spikes produced within a DP, the so-called quantal content or $Q_{\text{amp}}$) and the total number of spikes, considerably differed at the

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$n$, $N$</th>
<th>Peak $I_{Ca}$, pA</th>
<th>$Q_{Ca}$, pC</th>
<th>$\tau_{a}$, ms</th>
<th>$\tau_{r}$, ms$^{-1}$</th>
<th>$\tau_{d}$, ms$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>22°C</td>
<td>13, 3</td>
<td>320 ± 55</td>
<td>12.95 ± 1.78</td>
<td>1.11 ± 0.13</td>
<td>24.24 ± 1.98</td>
<td>0.76 ± 0.11</td>
</tr>
<tr>
<td>22°C + KBR</td>
<td>13, 3</td>
<td>302 ± 51ns</td>
<td>12.35 ± 1.72ms</td>
<td>1.06 ± 0.14ns</td>
<td>30.76 ± 3.84ms</td>
<td>0.89 ± 0.07ns</td>
</tr>
<tr>
<td>37°C</td>
<td>10, 2</td>
<td>552 ± 65*</td>
<td>19.18 ± 2.66*</td>
<td>0.43 ± 0.18*</td>
<td>28.49 ± 3.34</td>
<td>1.49 ± 0.30*</td>
</tr>
<tr>
<td>37°C + KBR</td>
<td>10, 2</td>
<td>499 ± 54ms</td>
<td>17.81 ± 2.15ms</td>
<td>0.31 ± 0.04ms</td>
<td>29.32 ± 5.23ms</td>
<td>1.89 ± 0.37ms</td>
</tr>
</tbody>
</table>

Data are means ± SE and were pooled and analyzed from the peak Ca$^{2+}$ current ($I_{Ca}$) obtained at −10 mV, shown in Fig. 1. $Q_{Ca}$, total Ca$^{2+}$ entry; $\tau_{a}$, time constant for current activation; $\tau_{r}$, time constant for current inactivation; $\tau_{d}$, time constant for current deactivation; $n$, number of cells; $N$, number of cultures. *$P < 0.05$, †$P < 0.001$, with respect to 22°C; ns, nonsignificant with respect to 37°C; *ns1, nonsignificant with respect to 22°C.

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two temperatures. For instance, pool analysis of 12 cells at 22°C
gave values of 18.3 ± 2.0 spikes and Qamp of 45.6 ± 7.5 pC; at
37°C (12 cells) these values rose to 67.2 ± 11.4 spikes and 126.1 ±
22.2 pC, threefold higher (Fig. 3, C and D). Although less drastic some differences were also found at
the level of single-spike analysis (Table 3). For instance, rise
rate and spike amplitude (Imax) were, respectively, 50 and 40%
higher at 37°C and spike duration was 26% smaller at 37°C

Table 2. Influence of temperature and KBR on the kinetic parameters of K+–elicited [Ca2+]c transients

<table>
<thead>
<tr>
<th></th>
<th>Amplitude, %</th>
<th>Width, %</th>
<th>Area, %</th>
<th>Rise Time, s (activation)</th>
<th>Rise Time, s (decay)</th>
<th>Decay Rate, s (decay)</th>
<th>Decay Rate, s (decay)</th>
<th>n, N</th>
</tr>
</thead>
<tbody>
<tr>
<td>22°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3 (control)</td>
<td>110.5 ± 3.3</td>
<td>105.0 ± 4.3</td>
<td>105.3 ± 5.4</td>
<td>0.71 ± 0.1</td>
<td>0.106 ± 0.006</td>
<td>6.9 ± 0.4</td>
<td>10.1 ± 0.6</td>
<td>19, 3</td>
</tr>
<tr>
<td>P4 (KBR)</td>
<td>104.5 ± 4.0</td>
<td>103.4 ± 5.0</td>
<td>104.0 ± 6.3</td>
<td>0.71 ± 0.14</td>
<td>0.108 ± 0.005</td>
<td>6.7 ± 0.3</td>
<td>9.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3 (control)</td>
<td>98.0 ± 1.1</td>
<td>101.7 ± 2.3</td>
<td>110.4 ± 4.3</td>
<td>0.69 ± 0.08</td>
<td>0.080 ± 0.009</td>
<td>9.7 ± 1.3</td>
<td>14.0 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>P4 (KBR)</td>
<td>72.7 ± 4.1</td>
<td>112.1 ± 5.7</td>
<td>78.5 ± 2.8</td>
<td>1.14 ± 0.08</td>
<td>0.042 ± 0.028</td>
<td>18.5 ± 2.5</td>
<td>26.8 ± 3.6</td>
<td>12, 3</td>
</tr>
</tbody>
</table>

Kinetic parameters were calculated from the transients generated by pulse P3 (controls) and P4 (KBR, 3 mM) of experiments done with the protocols shown
in Fig. 2. Data are means ± SE of the number of cells shown in n. Only P3 (control) and P4 (in the presence of KBR) were analyzed; the rest of control and
KBR peaks were quite similar to P3 and P4, respectively. Data of peak amplitude and area were normalized as % of P1 within each cell. Significant percent changes (decrease or increase) observed in cells exposed to KBR at 37°C are indicated in parentheses. [Ca2+]c, cytosolic Ca2+ concentration. *P < 0.01, †P < 0.001, with respect to P3 (control) at 37°C.
compared with the values obtained at 22°C. This is in agreement with quantal size (Q) that was 33% higher at 37°C with respect to 22°C. No differences were observed in spike decay parameters. Model spikes at 22 and 37°C were drawn on the basis of the mean kinetic parameters of Table 3; at 37°C the secretory event had a faster rise rate and peak amplitude, compared with the event at 22°C (Fig. 3E).

The effects of 3 μM KBR were tested with a protocol similar to that used for exploring its effects on [Ca²⁺]c transients. KBR was cell perifused during 2 min before and along P2, as indicated by horizontal lines at bottom. C and D: pooled results (means ± SE) of the number of cells and cultures shown in parentheses. They represent the cumulative total secretion per pulse (Qamp in pC, ordinates at left) and spike number secreted per DP (ordinates at right). **P < 0.001, with respect to P1. E: averaged spike profiles at 22 and 37°C.

Fig. 3. Effects of temperature and KBR on the quantal release of catecholamines amperometrically monitored with a carbon fiber microelectrode. After carbon fiber positioning, the selected cell was continuously perifused with a Tyrode solution containing 2 mM Ca²⁺. After baseline stabilization, the cell was challenged with 3 sequential DPs (P1, P2, and P3) of 10-s duration given at 5-min intervals with a solution containing 30 mM K⁺ and low Na⁺ containing 2 mM Ca²⁺ (30K⁺) at 22°C (A and C) and 37°C (B and D). A and B: original recordings of quantal catecholamine release of example cells at 22 and 37°C, respectively. KBR (3 μM) was present since 2 min before and during P2, as indicated by horizontal lines at bottom. C and D: pooled results (means ± SE) of the number of cells and cultures shown in parentheses. They represent the cumulative total secretion per pulse (Qamp in pC, ordinates at left) and spike number secreted per DP (ordinates at right). **P < 0.001, with respect to P1. E: averaged spike profiles at 22 and 37°C.

Table 3. Effects of temperature on the kinetics of quantal release of catecholamine triggered by 10-s 30K⁺ depolarizing pulses

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Cell Number</th>
<th>Spike Number</th>
<th>Rise Rate, pA/ms</th>
<th>Iₘₐₓ, pA</th>
<th>t₁/₂, ms</th>
<th>Fall 75–25, ms</th>
<th>Duration, ms</th>
<th>Q, pC</th>
</tr>
</thead>
<tbody>
<tr>
<td>22°C</td>
<td>17</td>
<td>286</td>
<td>30.62 ± 5.10</td>
<td>77.55 ± 9.53</td>
<td>13.08 ± 1.65</td>
<td>12.52 ± 1.81</td>
<td>73.00 ± 12.15</td>
<td>1.16 ± 0.13</td>
</tr>
<tr>
<td>37°C</td>
<td>10</td>
<td>361</td>
<td>46.66 ± 9.40</td>
<td>117.8 ± 14.67*</td>
<td>12.09 ± 1.57</td>
<td>10.97 ± 1.16</td>
<td>54.17 ± 4.91</td>
<td>1.55 ± 0.28</td>
</tr>
</tbody>
</table>

Data are means ± SE. Kinetic parameters of single secretory events were calculated from the P1 control responses of experiments shown in Fig. 3. 30K⁺, solution containing 30 mM K⁺. Iₘₐₓ, spike amplitude; Q, quantal size. *P < 0.05, compared with 22°C.
At 22°C, total secretion elicited by a 100K^+ DP was 1.25 ± 0.095 nC at 22°C and 3.66 ± 0.22 nC at 37°C (n = 70 DPs). This threefold higher response at 37°C was similar to that observed when measuring quantal release at the single-cell level (Fig. 3). At 22°C these responses underwent ~20% decay upon repeated K^+ pulsing (control curves in Fig. 4B). However, the responses were well maintained at 37°C (Fig. 4A).

KBR at 1 mM produced 50% blockade of secretion at 37°C (Fig. 4A); however, the compound did not alter the responses at 22°C (Fig. 4B). In contrast, the K^+ responses were halved by 3 μM nifedipine at both temperatures (Fig. 4, A and B). This blockade was quantitatively similar to that obtained in previous studies also performed in BCCs (23, 37). When added together, the response was further depressed by 15% (Fig. 4, E and F). It should be noticed that non-L nifedipine-resistant PQ channels contribute ~50% to the K^+ secretory response in BCCs at room temperature (23).

Slightly different experiments were performed with FPL (3 μM) that was combined with 1 mM KBR. At 37°C KBR depressed secretion by 50%. Subsequently and always in the presence of KBR, FPL still enhanced the secretory response to 72.8% of the initial response (Fig. 4C). Again at 22°C KBR did not block secretion; however, when added in the presence of KBR, FPL still enhanced exocytosis by 61.6% above control (Fig. 4D).

Because at 37°C both nifedipine and KBR blocked by ~50% the secretory response, the question rose on whether the combined cell treatment with both compounds could elicited a full blockade. In the example experiment of Fig. 4E, the initial cell perfusion with KBR halved the K^+ secretory responses. Later addition of nifedipine caused a mild further depression. Pooled results from six experiments are graphed in Fig. 4F, showing that combined nifedipine and KBR did not elicit full blockade of the response. It is worthy of comment the fact K^+ secretion in perfused BCC populations has a nifedipine-resistant component that is controlled by Ca^{2+} entry through PQ-type VDCCs (23).

**Effects of temperature, KBR, and SEA on secretory responses elicited by caffeine or histamine.** In BCCs transfected with aequorins targeted to the endoplasmic reticulum (ER), we previ-
ously demonstrated the fast decline of the ER Ca\(^{2+}\) concentration elicited by caffeine or histamine (1). The ensuing elevation of the [Ca\(^{2+}\)]\(_{\text{c}}\) triggers the release of catecholamines in BCCs stimulated with caffeine or histamine (26). As described above, KBR inhibited the secretion stimulated by Ca\(^{2+}\) entry through VDCCs opened by a DP at 37°C but not at 22°C. It was therefore of interest to explore whether a similar behavior appeared on the secretory response elicited by caffeine or histamine that causes the release of Ca\(^{2+}\) from the ER in BCCs (1).

These experiments had a design similar to those of K\(^{+}\) (Fig. 4); however, here 2-s pulses of caffeine (50 mM) or histamine (100 \(\mu\)M), instead of K\(^{+}\), were used to stimulate the release of catecholamine. Thus repeated pulsing with caffeine evoked secretory spikes of decreasing magnitude as shown in the example experiment of Fig. 5A in cells superfused at 37°C (P1, P2, P3). It was surprising that in the presence of 3 \(\mu\)M KBR the secretory spike (P4) was nearly threefold higher than P3.

**Fig. 5.** Effects of temperature and KBR on the secretory responses triggered by caffeine or histamine in populations of BCCs at 37 and 22°C. Cells were continuously perifused with a Krebs-HEPES solution containing 2 mM Ca\(^{2+}\). After a 10-min equilibration period, they were challenged with sequential 2-s pulses of 50 mM caffeine (A–C) or 100 \(\mu\)M histamine (D–F). A: example amperometric spike recording of catecholamine release triggered by 4 caffeine pulses (P1 to P4) given to cells superfused at 37°C; P4 was given in the presence of 3 \(\mu\)M KBR that was superfused since 2 min before and during P4, as indicated by the horizontal bar at bottom. B: pooled results on the total secretion per caffeine pulse (spike area in \(\mu\)C, ordinate). C: caffeine experiments performed at 22°C. D: example amperometric secretory spike records obtained from cells challenged with repeated histamine pulses (P1 to P4); P4 was given in the presence of 3 \(\mu\)M KBR (horizontal bar at bottom). E: pooled results of histamine pulses given in the absence (P3, control) and the presence of KBR (P4). F: similar histamine experiments performed at 22°C. Spike areas (total secretion in \(\mu\)C, ordinates) in B, C, E, and F are means ± SE of the number of experiments and cultures shown in parentheses. *\(P < 0.05\), **\(P < 0.01\), compared with controls (Ctrl).

Pooled data from 10 experiments showed that the augmentation of secretion in the presence of KBR was over twofold (Fig. 5B). Similar experiments performed at 22°C gave quite different results. Caffeine produced a quantitative secretory response similar to that seen at 37°C (1.4 \(\mu\)C of catecholamine). How-
ever, in the presence of KBR the response was unchanged, 1.5 μC of catecholamine (Fig. 5C). In another series of experiments, secretion was triggered by 2-s pulses of histamine given at 4-min intervals. In the example experiment of Fig. 5D performed in cells perfused at 37°C, the three first histamine pulses (P1 to P3) gave quite reproducible secretory responses of around 200 nA amplitude. In the presence of 3 μM KBR, the histamine response was enhanced by 2.5-fold (Fig. 5D, P4). In 10 experiments the mean potentiation by KBR of the histamine response was 2.5-fold (Fig. 5E).

At 22°C the histamine secretory response was 1.4 μC, a figure slightly smaller than that obtained at 37°C. KBR (3 μM) augmented such response to 1.9 μC, a figure substantially smaller than the value obtained at 37°C (P < 0.01).

Experiments following the caffeine protocol at 37°C were additionally performed with compound SEA, a more selective and potent blocker of the NCX (25). Five-second pulses of 20 mM caffeine elicited secretory responses of 300 nA. Secretion rose to near 500 nA in the presence of 1 μM SEA; this response remained high during the three sequential pulses of caffeine and returned to baseline upon SEA withdrawal (Fig. 6A). Of note was the higher width of the secretion responses in the presence of SEA (arrows in Fig. 6, A and B), indicating that total secretion (spike area) was increased by threefold (Fig. 6C).

Inhibition by KBR of K⁺-evoked catecholamine release from cell populations perfused at 37°C as a function of DP duration and the external Ca²⁺ concentration. We wanted to explore next whether the degree of blockade exerted by KBR on the K⁺-evoked secretion at 37°C was dependent of the duration of the K⁺ DP. To this aim, after 5-min equilibration the cells were challenged with pulses of increasing length (1–5 s) at 1-min intervals with a solution containing 100 mM K⁺, low Na⁺, and 2 mM Ca²⁺ (100K⁺/2Ca²⁺ solution). This high K⁺ content of the depolarizing solution was required to achieve a measurable caffeine elicited secretory responses of ~300 nA. Secretion rose to near 500 nA in the presence of 1 μM SEA; this response remained high during the three sequential pulses of caffeine and returned to baseline upon SEA withdrawal (Fig. 6A). Of note was the higher width of the secretion responses in the presence of SEA (arrows in Fig. 6, A and B), indicating that total secretion (spike area) was increased by threefold (Fig. 6C).

Fig. 7. KBR blocks the K⁺-evoked catecholamine release from BCCs superfused at 37°C. Prototype experiments shown in A and B were performed in 2 separate batches of cells from the same culture. After a stable secretion baseline was reached cells were stimulated with a Krebs-HEPES solution containing 100 mM K⁺ and 2 mM Ca²⁺ (100K⁺/2Ca²⁺ solution), with pulses of increasing length (1–5 s) at 1-min intervals. Two series of 100K⁺/2Ca²⁺ pulses were applied 10 min apart in the experiment of A. In the experiment of B, 3 μM KBR was present 4 min before and during the application of the second series of 100K⁺/2Ca²⁺ pulses (horizontal black bar at bottom). C: blockade by KBR of K⁺-evoked catecholamine release was readily reversible. In these experiments, cells were stimulated with 2-s pulses of 100K⁺/2Ca²⁺ solution at 1-min intervals and 37°C. KBR was given as shown by the horizontal bars at bottom. Total secretion in each spike of every individual experiment was calculated as spike area (μC), and was expressed as % of the maximum response obtained in the 1st pulse. Data are means ± SE of the number of experiments and cell cultures indicated in parenthesis. **P < 0.01, with respect to secretion obtained before addition of KBR.
able amperometric catecholamine release signal at the shorter (1–2 s) DP duration. The K+ DPs evoked secretory spikes of increasing magnitude, from ~100 nA with the 1-s pulse to ~500 nA with the 5-s pulse (Fig. 7A).

After this series of DPs, cells were left in resting conditions for a 10-min period and then the series of DPs of increasing duration were repeated once more; the secretory spikes of this second series had amplitudes similar to the first (example experiment of Fig. 7A). In a different batch of cells, the second series of DPs were applied in the presence of 3 μM KBR (Fig. 7B), given 4 min before and during the 5-min duration of the second series of DPs. Of note was the fact that secretion augmented gradually with DPs of 1–3-s duration but spikes had about half of the magnitude of the 1- to 3-s spikes of the control first DP series. At longer DPs (4–5 s), the responses did not augment further and in fact tended to be smaller than those elicited by the 3-s DP so that KBR blocked the responses by ~80%.

In the experiments of Fig. 7C, cells were repeatedly stimulated with 100K+ DPs of 3-s duration given at 1-min intervals. The initial averaged total secretion was 3.93 ± 0.25 μC (n = 22 DPs, 10 batches of cells from 3 different cultures). At 3 μM, KBR reduced this response by 51.2%; upon washout the response recovered to 84% of initial. The reintroduction of the compound twice more produced a similar re-blockade of catecholamine release (47.4%) as well as its recovery upon washout of KBR (to 74% of the initial response).

Fig. 8. Blockade by increasing concentrations of KBR of the catecholamine secretory responses evoked by K+ pulses of increasing length (abscissae) in a low (1 mM) or high (10 mM) extracellular Ca2+ concentration ([Ca2+]e). Secretion was studied following the protocols described in Fig. 7; all superfusing solutions were at 37°C. Areas of each secretory spike were calculated in each individual experiment (total secretion in μC); the maximum response (that obtained during the 5-s pulse of the first series of K+ pulses) was normalized to 100%, and the rest of the pulses expressed as % of such maximum response (ordinates). A–D: experiments performed in 1 mM [Ca2+]e; E–H: experiments performed in 10 mM [Ca2+]e. KBR was present during the second series of pulses at the concentrations indicated. Data are means ± SE of the number of experiments shown in parentheses from at least 3 different cell cultures. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the same K+-pulse duration in the absence of KBR.
In 11 additional experiments, cells were stimulated with 5-s DPs of a solution containing 35 mM K+ instead of 10 mM K+. In control cells, the initial total secretion was 2.15 ± 0.3 μC; KBR reduced the response to 1.21 ± 0.18 μC. This near 50% blockage suggests that the Na+ concentration of the K+ depolarizing solutions (40 mM in the 100K+ solution and 105 mM in the 35K+ solution) was not influencing the inhibitory effects of KBR on K+-evoked secretion.

Experiments with protocols similar to those of Fig. 7A were performed in cells perfused at 37°C with Krebs-HEPES solutions containing 1 or 10 mM Ca2+. Control responses were similar at both Ca2+ concentrations (Fig. 8, A and E) namely 0.96 ± 0.16 μC with 1-s 100K+ DPs at 1 mM Ca2+ (n = 24 cell batches) and 0.95 ± 0.12 μC at 10 mM Ca2+ (n = 28 cell batches). In the case of 5-s DPs, secretion was 4.46 ± 0.85 μC (1 mM Ca2+) and 5 ± 0.4 μC (10 mM Ca2+). At 1 mM Ca2+, secretion was drastically inhibited by 1, 3, and 10 μM KBR; however, the blockade was smaller with 1- to 2-s pulses compared with longer pulses (3–5 s; Fig. 8, B–D). This dependence on DP duration of KBR blockade was clearer at 10 mM Ca2+. Thus, at 1 μM, significant blockade was achieved only at 4- to 5-s DPs. At 3 and 10 μM KBR, the blockade of secretion exerted by KBR was significant at DPs of 3–5-s duration; at 1–2-s DPs KBR did not block the 100K+-evoked secretory responses (Fig. 8, F–H). It seems therefore that the effect of KBR depends on the time of Ca2+ entry as well as on the amount of total Ca2+ entering the cell during a given DP.

**DISCUSSION**

The central finding of this investigation was the blockade of exocytosis by KBR at 37°C but not at 22°C. Since this compound is reputed as a blocker of the NCX, the question is whether its temperature-dependent effects on the K+-elicted BCC secretory responses are linked to the transporter. If so, the second relevant question is related to the mechanism underlying its inhibitory effects on exocytosis. A final question addresses the physiological implications of such effects.

Higher rates of exocytosis at 37°C compared with lower temperatures have been previously reported to occur in BCCs (5, 15, 16, 18, 38). Here we have also found a threefold increase in cumulative secretion at 37°C with respect to 22°C (Fig. 3). The kinetic parameters of single-spike events indicated a faster fusion pore expansion and decay of released catecholamine at 37°C compared with 22°C (Fig. 3E); however, differences in various other kinetic parameters were at the limit of statistical significance (Table 3).

Temperature dependence of ion fluxes through a carrier considerably differs from those taking place through an ion channel. For instance, in chromaffin cells the NCX activity is fivefold higher at 37°C (36) than at 27°C (32). In contrast, the conductance of ion channels is relatively insensitive to temperature, having a Q10 of 1.2–1.5 (13); this conductance is like that for aqueous diffusion of ions and for the reciprocal of water viscosity. In the context of distinct temperature dependence of ion movements through the NCX and ion channels, two observations in this study could find a plausible explanation. The first concerns the scarce differences between \( I_{Ca} \) monitored at 22 and 37°C; an apparent greater peak amplitude (\( P < 0.05 \)) and faster activation at 37°C cannot explain the fact KBR did not affect current size and kinetics at both temperatures (Fig. 1 and Table 1). The second observation is associated to the different temperature dependence of the effects of KBR, nifedipine, and FPL on the K+-elicted bulk secretion of catecholamine. While blockade by KBR of such response was present at 37°C but not at 22°C, the blockade exerted by the L-type VDCC inhibitor nifedipine was similar at both temperatures (Fig. 4, A and B). Furthermore, the L-type VDCC activator FPL augmented the secretory responses at both temperatures even in the presence of KBR, indicating a different site of action for KBR and the ligands of L channels. The fact KBR altered the exocytotic signals at 37°C but not at 22°C, and nifedipine and FPL altered the signals at both temperatures keeps pace with their targets namely the higher temperature dependence of NCX and the lower temperature-dependence of L-type VDCCs, respectively.

![Fig. 9. Scheme to illustrate how the forward mode of the Na+/Ca2+ exchanger (rNCX) and its reverse mode (nNCX) may considerably contribute to shaping the cytosolic elevations of the free Ca2+ ion concentrations ([Ca2+]c) and the catecholamine release responses, occurring during chromaffin cell depolarization at 37°C. During a DP Ca2+ entry from the extracellular space ([Ca2+]e) occurs via voltage-dependent Ca2+ channels (VDCCs) and the rNCX (A); the longer the DP, the greater the contribution of the rNCX to Ca2+ entry because of VDCC inactivation. After the DP ends (B), Ca2+ entry ceases and the rNCX contributes to restore the basal [Ca2+]c, (Ca2+ clearance), C: model of [Ca2+]c elevation and clearance during and after a DP (A and B), and the contribution of the nNCX to [Ca2+]c, rise, and of nNCX to [Ca2+]c, clearance.](http://ajpcell.physiology.org/)
Additional evidence in support of the supposition that temperature dependence of KBR effects on secretion is linked to blockade of the NCX comes from the experiments with caffeine and histamine. These two agents are known to elevate \([\text{Ca}^{2+}]_c\), as a result of its release from the ER, thus causing the secretion of catecholamine from BCCs (1, 19). Contrarily to inhibition of the K⁺ response, KBR enhanced the secretion responses of caffeine and histamine at 37°C (Fig. 5, B and E), and similarly to the K⁺ responses, KBR did not affect the histamine- and caffeine-evoked secretion at 22°C (Fig. 5, C and F). The simplest explanation for this finding relies on the supposition that KBR effects are visible only when the NCX is maximally working at 37°C. At this temperature, the forward mode of the NCX could substantially contribute to the \([\text{Ca}^{2+}]_c\) transient elicited by caffeine and histamine, thus limiting their secretory responses; mitigation of \(\text{Ca}^{2+}\) efflux by KBR will slow down the \([\text{Ca}^{2+}]_c\) clearance to enhance secretion. Of note was the observation that the more selective and potent NCX blocker compound SEA caused even a more augmented caffeine-elicited secretion response (Fig. 6) compared with KBR (Fig. 5A) at 37°C. This supports our view that at physiological temperature the forward mode of the NCX greatly contributes to \([\text{Ca}^{2+}]_c\), clearance and secretion elicited by ER \(\text{Ca}^{2+}\) release or during the few-second period following a DP, as discussed next.

The physiological role of the NCX at shaping the kinetics of \([\text{Ca}^{2+}]_c\) transients (and hence of secretory responses) is schematically represented in Fig. 9. During a DP, \(\text{Ca}^{2+}\) enters the cell through VDCCs and the rNCX; this reverse mode of the exchanger is operative during cell depolarization (6). Thus a large and fast elevation of \([\text{Ca}^{2+}]_c\) is produced (Fig. 9A). In the few-second period following the DP, the forward mode of the exchanger contributes to \(\text{Ca}^{2+}\) clearance (Fig. 9B). Overall, this high and rapid \([\text{Ca}^{2+}]_c\) transient (Fig. 9C) gives rise to a large secretory response. In the presence of KBR, the exchanger is working at fivefold lower rate. Hence, the \([\text{Ca}^{2+}]_c\) elevation is taking place only at the expense of \(\text{Ca}^{2+}\) entry through VDCCs (Fig. 9D), which in BCCs are known to undergo voltage- and \(\text{Ca}^{2+}\)-dependent inactivation in the time span of few seconds (17, 37). This could explain that during the 10-s DP when the rNCX is blocked by KBR, the mitigated rate of \(\text{Ca}^{2+}\) entry only through those VDCCs produces a lower-amplitude \([\text{Ca}^{2+}]_c\) transient (Fig. 9F). Furthermore, during the post-DP period the contribution of the rNCX is also decreased and hence the \([\text{Ca}^{2+}]_c\) clearance is markedly slowed down (Fig. 9, E and F). This milder and slower \([\text{Ca}^{2+}]_c\) transient is therefore producing a smaller secretory response. Thus it seems that the pronounced secretory response to few-second depolarization of BCCs at 37°C is elicited by \(\text{Ca}^{2+}\) entry through both VDCCs and the rNCX.

This mechanistic explanation also fits with the secretion blockade by KBR with longer (5–5 s) but not with shorter DPs (Fig. 8). Earlier in a DP (for instance, 1 s), the large electrochemical gradient for \(\text{Ca}^{2+}\) entry through VDCCs (500 μmol·l⁻¹·s⁻¹) (2, 41) overwhelms, the \(\text{Ca}^{2+}\) entry component through the rNCX; thus, although smaller than with longer 3- to 5-s pulses, the secretory response elicited by 1- to 2-s pulses is dominated by \(\text{Ca}^{2+}\) entering the cell through VDCCs; it then follows that the blockade of the rNCX by KBR will not be visible. However, as discussed above the longer DPs leads to VDCC inactivation; in this manner, the contribution of the rNCX to \(\text{Ca}^{2+}\) entry and the overall secretory response will be more relevant and hence KBR will elicit its blockade.

From a physiological point of view we may try to discern how the results of this investigation fit in the physiological function of the sympathoadrenal axis. Under resting conditions, the sympathetic cholinergic nerves to the adrenal medulla chromaffin cells fire at ~0.2 Hz. During a situation of fear, alarm, stress, or conflicts, the sympathoadrenal axis fire nerve impulses at 10–20 Hz or more to discharge a massive secretion of catecholamine (10). In this last situation is where the NCX could play a relevant role in maintaining and securing a noninactivating, high rate of catecholamine release to mobilize the body for the “fight or flight” response that prepares the animal for immediate fight or to run away from danger (4, 7). The way the NCX could perform such task is by contributing to \(\text{Ca}^{2+}\) entry in its reverse mode under the high-firing rate of sympathoadrenal medullary axis, a situation of prolonged cell depolarization that may lead to partial inactivation of VDCCs of chromaffin cells and to a predominance of \(\text{Ca}^{2+}\) entry through the rNCX.

In conclusion, we provide here experimental support for a relevant role of the NCX in shaping the \([\text{Ca}^{2+}]_c\) and catecholamine release transients triggered by K⁺ DPs of few-second duration applied to BCCs at 37°C. Although previous studies mostly performed at room temperature with long-period treatments with ouabain and ionic manipulations have implicated the NCX in triggering the release of catecholamine, our present study is the first supporting the view for a role of the NCX under physiological temperature and chromaffin cell depolarization, in the short-term control (few seconds) of catecholamine release.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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Biological processes such as neurotransmitter release are complex and involve multiple components acting in concert. The sodium-calcium exchanger (NCX) is a key player in these processes, mediating the exchange of sodium and calcium across the plasma membrane. The study by Frankenhaeuser and Moore (1963) demonstrated the role of temperature on sodium-calcium exchange in bovine chromaffin cells, highlighting the importance of this process in physiological homeostasis.

Further research by Frankenhaeuser and Moore (1963) also showed that the activity of the calcium pump, which is another critical element in calcium homeostasis, is influenced by temperature. This study underscores the significance of temperature in the regulation of calcium levels within cells.

The role of the NCX in neurotransmitter release was also explored by Liu and colleagues (2001), who examined the effect of ouabain on the exchanger's activity. Their findings contribute to our understanding of how the NCX responds to pharmacological interventions and how these responses might be harnessed for therapeutic purposes.

The study by de Diego et al. (1992) on the expression of the NCX in chromaffin cells is significant as it provides insights into the cellular mechanisms underlying neurotransmitter release. This work has implications for the development of treatments aimed at modulating neurotransmitter release.

Collectively, these studies illustrate the intricate nature of calcium homeostasis and the manifold roles the NCX plays in this process. They also underscore the importance of temperature and pharmacological interventions in modulating calcium fluxes, which are essential for understanding and managing neurological and cardiovascular disorders.