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Direct modulation of volume-regulated anion channels by Ca^{2+} chelating agents

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Abstract **Ca^{2+} chelating agents are widely used in biological research for Ca^{2+} buffering. Here we report that BAPTA, EDTA and HEDTA produce fast, reversible, voltage-dependent inhibition of swelling-activated Cl^- current ($I_{Cl,swell}$) in LNCaP prostate cancer epithelial cells that is unrelated to their Ca^{2+} binding. BAPTA was the most effective (maximal blockade 67%, $IC_{50} = 70$ μM, at +100 mV) followed by EDTA and HEDTA. $I_{Cl,swell}$ blockade by EDTA was pH-dependent. BAPTA blocked $I_{Cl,swell}$ also in other cell types. We conclude that Ca^{2+} chelating agents block $I_{Cl,swell}$ by acting directly on the underlying channel, and that the negative charge of the free chelator form is critical for the blockade.** © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: LNCaP prostate cancer cell; Volume-regulated anion channel; Swelling-activated chloride current; Calcium chelating agent; BAPTA; EDTA

1. Introduction

Ca^{2+} is a universal second messenger regulating many cellular processes and a number of ion channels. The special regulatory role of this vital and most abundant divalent macro-element is realized via its ability to form high affinity coordination bonds with various anionic groups of the proteins lowering its effective regulatory concentrations well below micromolar levels. Therefore, identification of high affinity Ca^{2+} regulatory sites and uncovering their physiological roles often requires the use of Ca^{2+} chelating agents (EGTA, EDTA, BAPTA, etc.) in order to permit sufficient lowering and controlled manipulation of its concentration. Traditionally Ca^{2+} chelating agents were considered to be neutral producing effects only via changing free Ca^{2+} concentration. Since physiological Ca^{2+} concentrations inside the cell ($[Ca^{2+}]_{in}$) are within the submicromolar range, which is at least 1000 times lower than outside, the most common use of Ca^{2+} chelating agents is to buffer free Ca^{2+} concentrations ($[Ca^{2+}]_{free}$) in artificial intracellular salines. However, experimental needs often require lowering the extracellular Ca^{2+} concentrations ($[Ca^{2+}]_{out}$) as well. As far as Ca^{2+} regulation of membrane ion channels is concerned, the brightest example of the productivity of this approach was the discovery of Ca^{2+}-dependent permeability switch of voltage-gated [1,2] and store-operated Ca^{2+} channels (SOCs) [3,4] resulting in their ability to pass huge Na^+ current under conditions of submicromolar extracellular Ca^{2+}.

Volume-regulated anion channels (VRACs), which carry swelling-activated Cl^- current ($I_{Cl,swell}$), are widely represented in many cell types, where they play a key role in regulatory volume decrease [5–7]. We have recently described VRAC-carried $I_{Cl,swell}$ in the human prostate cancer epithelial cell line LNCaP (lymph node carcinoma of the prostate) [8], and showed its susceptibility to the regulation by Ca^{2+} entering the cell via plasma membrane SOCs [9]. Here, by focusing on possible modulation of $I_{Cl,swell}$ in LNCaP cells by submicromolar concentrations of extracellular Ca^{2+} we unexpectedly discovered that Ca^{2+} chelating agents, such as BAPTA, EDTA and HEDTA, used to buffer $[Ca^{2+}]_{out}$, are themselves capable of inducing voltage-dependent $I_{Cl,swell}$ inhibition. This inhibition appeared to be not restricted to LNCaP cells, but characteristic of all VRACs in general irrespective of their cell-specific phenotypic representation. Our data suggest the existence in VRAC of an interaction site with Ca^{2+} chelating agents and prompts careful selection of the type and concentration of the chelator for Ca^{2+} buffering purposes.

2. Materials and methods

The procedures of LNCaP cells (American Type Culture Collection) culturing and preparing them for electrophysiology are detailed elsewhere [8]. Macroscopic membrane currents were recorded using the patch-clamp technique in its whole-cell configuration as described previously [8]. To prevent $I_{Cl,swell}$ contamination with LNCaP cell endogenous K^+ current [10] we used TEA as a major cation in the hypotonic solution. The composition of the normal (310 mosmol/l) and TEA-based hypotonic (200 mosmol/l, hypo-TEA) extracellular solutions is presented in Table 1. Concentration of Ca^{2+} chelators (i.e. BAPTA, EDTA or HEDTA) in the divalent cation-free hypo-TEA (DVC-free/hypo-TEA) was changed in parallel with D-mannitol to keep the osmolarity constant. The free Ca^{2+} concentration in the presence of Ca^{2+} chelators was determined with the use of WinMaxc 1.7 program [11]. The resistance of the patch pipettes fabricated from borosilicate glass capillaries (WPI, Saratoga, FL, USA) varied between 3 and 5 MΩ when filled with the basic intracellular solution containing (in mM): K-gluconate 100, KCl 50, $MgCl_2$ 1, $CaCl_2$ 2.6, HEPES 10, EGTA 8, pH 7.2, osmolarity 290 mosmol/l, estimated $[Ca^{2+}]_{free} = 10^{-7}$ M. Changes of the external solutions were carried out using a multibarrel puffing micropipette with common outflow positioned in close proximity to the cell under investigation.

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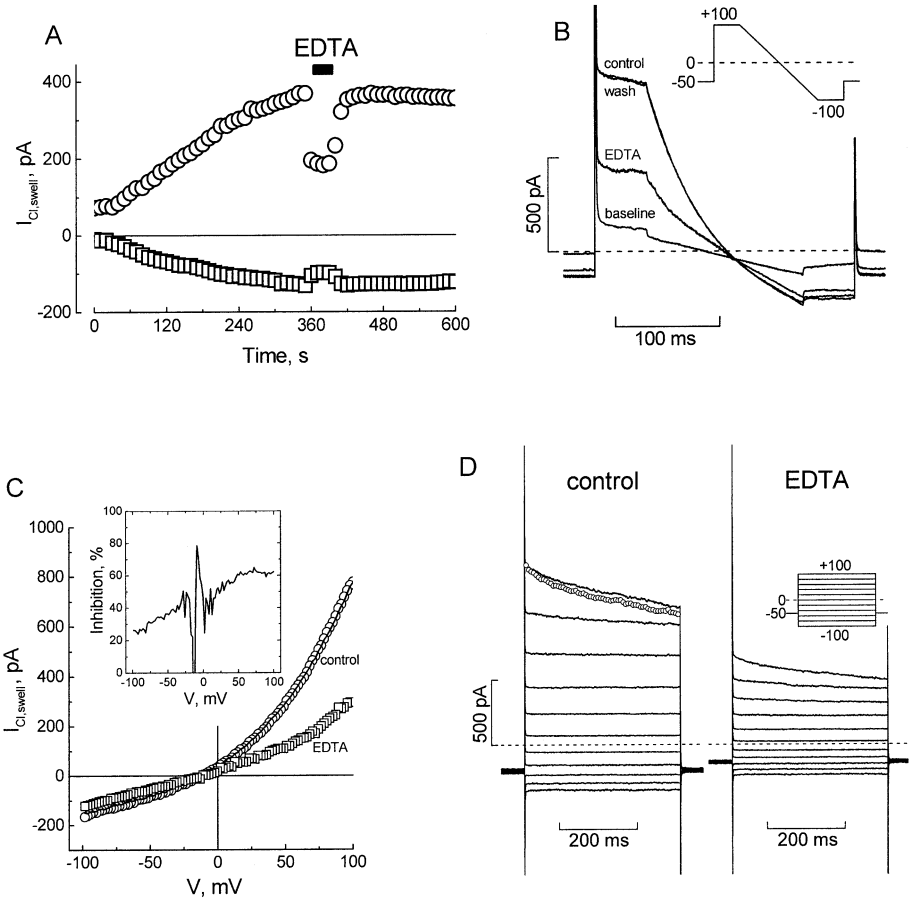

Fig. 1. Extracellular EDTA inhibits $I_{Cl,swell}$ in LNCaP cells. A: $I_{Cl,swell}$ development in response to hypotonic exposure (at time 0), and the effect on the current of 10 mM EDTA, (circles) $I_{Cl,swell}$ at +20 mV, (squares) at −50 mV; EDTA application is marked by horizontal bar. B: traces of the baseline current before $I_{Cl,swell}$ activation, control $I_{Cl,swell}$, EDTA-inhibited $I_{Cl,swell}$ and $I_{Cl,swell}$ following EDTA washout; inset: pulse protocol. C: *I–V* plots of the control $I_{Cl,swell}$ (circles), EDTA-inhibited $I_{Cl,swell}$ (squares) and $I_{Cl,swell}$ after EDTA washout (line superimposed on circles); the *I–V*s were derived from ramp recordings of B; inset: voltage dependence of inhibition. D: Representative step pulse-evoked (see inset) traces of the control $I_{Cl,swell}$ (left) and EDTA-inhibited $I_{Cl,swell}$ (right); the dotted line superimposed on the control trace at +100 mV represents the EDTA-modified trace normalized to the same amplitude at +100 mV demonstrating virtually no difference in the kinetics. The data on this figure are for one representative LNCaP cell.

3. Results

3.1. The effects of EDTA, BAPTA and HEDTA on $I_{Cl,swell}$ in LNCaP cells

Exposure of LNCaP cells to hypo-TEA (see Table 1) elicits the development of $I_{Cl,swell}$, which usually reaches its maximal amplitude within the time span of about 5 min (Fig. 1A). Fig. 1B shows representative recordings of the initial baseline current acquired within the first few seconds after switching to hypo-TEA and of fully developed $I_{Cl,swell}$ in response to the voltage-clamp pulse that included a linear voltage ramp portion connecting steady ±100 mV levels. The *I–V* relationship of $I_{Cl,swell}$ derived from the ramp portions of the currents showed outward rectification typical for $I_{Cl,swell}$ and a reversal potential close to the Cl^- equilibrium (Fig. 1C). Replacing standard hypo-TEA solution with DVC-free/hypo-TEA (see Table 1) supplemented with 10 mM EDTA caused almost instantaneous decrease of $I_{Cl,swell}$, which was much more pronounced at positive membrane potentials (Fig. 1A). The *I–V* relationship of the residual current (Fig. 1C) showed the same reversal potential as the control $I_{Cl,swell}$ indicating its Cl^- nature and suggesting that it represents a modified version of the original $I_{Cl,swell}$. The modification primarily consisted in the voltage-dependent inhibition of $I_{Cl,swell}$, the degree of which gradually increased from just around 20% at −100 mV up to about 50–60% at +50 mV without significant change from this level at higher depolarizations (Fig. 1C, inset). Kinetics of $I_{Cl,swell}$ at step pulses to various voltages remained almost unchanged (Fig. 1D).

To answer the question whether the modification of $I_{Cl,swell}$

Table 1
Composition of the extracellular solutions (mM)

	Normal	Hypo-TEA	DVC-free/hypo-TEA
NaCl	140	–	–
KCl	5	–	–
$CaCl_2$	2	2	0
$MgCl_2$	2	2	0
Na_2HPO_4	0.3	–	–
KH_2PO_4	0.4	–	–
$NaHCO_3$	4	–	–
Glucose	5	5	5
HEPES	10	10	10
TEA-Cl	–	80	88
D-Mannitol	–	14	10–0
Ca^{2+} chelator	–	–	0–40

pH of all solutions was adjusted to 7.2. As Ca^{2+} chelator BAPTA, EDTA or HEDTA was used.

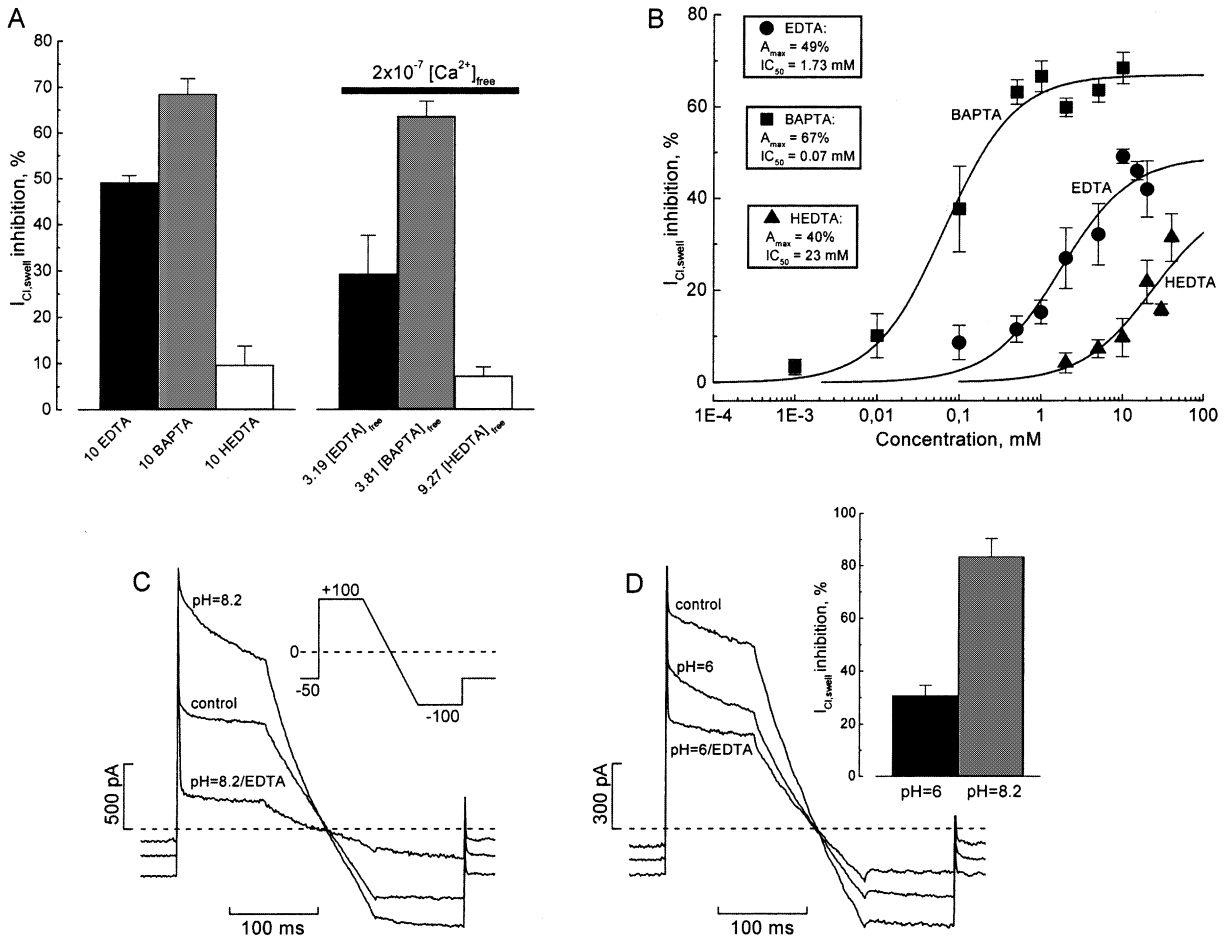

Fig. 2. $I_{Cl,swell}$ inhibition by Ca^{2+} chelating agents is unrelated to Ca^{2+} binding and is pH-dependent. A: $I_{Cl,swell}$ blockade at +100 mV by 10 mM of specified chelator (left) and by the indicated free concentration (mM) of the chelator in a Ca^{2+}/10 mM chelator mixture resulting in a fixed $[Ca^{2+}]_{free}$ of 2×10^{-7} M (right); $n=6$–10. B: Dose–response relationships for the BAPTA-, EDTA- and HEDTA-induced $I_{Cl,swell}$ inhibition with superimposed fits of the data points ($n=6$–10) by Langmuir's isotherms (smooth curves) providing indicated values of the maximal blockade (A_{max}) and IC_{50}. C: Traces of the control $I_{Cl,swell}$ at standard pH 7.3, $I_{Cl,swell}$ at increased pH of hypo-TEA to 8.2 and $I_{Cl,swell}$ in the presence of 10 mM EDTA at pH 8.2; inset: pulse protocol. D: Same as in C, but for the decreased pH 6; inset: the difference in $I_{Cl,swell}$ blockade by 10 mM EDTA at pH 6 and pH 8.2 ($n=6$).

is related to some specific actions of EDTA or is simply due to its ability to lower the concentration of the divalents we performed similar experiments with the use of two other Ca^{2+} chelating agents, BAPTA and HEDTA (*N*-[2-hydroxyethyl]ethylenediaminetriacetic acid, do not mix up with the monoprotonated form of EDTA designated below as $HEDTA^{3-}$). Fig. 2A compares the average $I_{Cl,swell}$ inhibition at +100 mV produced by 10 mM EDTA, BAPTA or HEDTA. As one can see, the percentage of current inhibition was the highest for BAPTA (67%), followed by EDTA (49%) and then HEDTA (35%). Theoretical estimates of the free Ca^{2+} concentration in the solutions containing 10 mM of the respective chelator showed that it constitutes 1.2×10^{-11} M for BAPTA, 2.5×10^{-12} M for EDTA and 2.5×10^{-10} M for HEDTA (assuming 10^{-6} M of residual Ca^{2+} in nominally Ca^{2+}-free solution confirmed by our control Fura-2 measurements). Thus, with any of the three chelators added $[Ca^{2+}]_{free}$ seems to drop far below physiologically relevant levels suggesting that $I_{Cl,swell}$ inhibition can hardly be explained by changing $[Ca^{2+}]_{out}$. Moreover, application of DVC-free/hypo-TEA solutions, in which $[Ca^{2+}]_{free}$ was buffered at a constant level of 2×10^{-7} M, but with the use of 10 mM BAPTA, EGTA or HEDTA, produced differential blockade of $I_{Cl,swell}$ (Fig. 2A). Again, this blockade was the strongest for BAPTA (65%) followed by EDTA (26%) and then HEDTA (7%) ruling out the involvement of Ca^{2+}. The blockade also did not correlate with the concentration of free chelator suggesting a great degree of specificity among them.

Given all the data stated above one can assume that direct specific actions of BAPTA, EGTA and HEDTA on volume-regulated channels may be responsible for $I_{Cl,swell}$ inhibition. Fig. 2B shows dose–response relationships for the inhibitory action of all three Ca^{2+} chelating agents on $I_{Cl,swell}$ at +100 mV. Because the Ca^{2+} chelator-induced $I_{Cl,swell}$ inhibition was very rapid (see Fig. 1A) we could use in these experiments very brief exposures of the cell to concentrations of EDTA or HEDTA higher than 10 mM (which could increase the tonicity of the solution) without fear of invoking the changes of the current associated with altered tonicity. The experimental data points were fit with Langmuir's isotherms (Fig. 2B) to provide the concentration of half-maximal blockade (IC_{50}) and the level of maximal blockade (A_{max}). The results of the fit show that BAPTA is the most effective $I_{Cl,swell}$ inhibitor acting with $IC_{50}=70$ μM and the maximal

blockade of $A_{max} = 67\%$. EDTA was next with $IC_{50} = 1.7$ mM and $A_{max} = 49\%$ followed by HEDTA with $IC_{50} = 23$ mM and $A_{max} = 40\%$.

3.2. Inhibition of $I_{Cl,swell}$ by EDTA is pH-dependent

Free EDTA in the solution exists as negatively charged partially protonated species with pH-dependent contribution of various forms [12]. At physiological pH most of it is in $HEDTA^{3-}$ form with a quite low contribution of the H_2EDTA^{2-} and $EDTA^{4-}$ forms and only negligible traces of all other ones. The proportion of the $EDTA^{4-}$ form, however, significantly increases at the expense of H_2EDTA^{2-} form, as pH is made more alkaline, whereas the H_2EDTA^{2-} form becomes absolutely dominant at $pH < 6$. Since VRAC is an anion channel it seems plausible that the preferential EDTA-induced blockade of $I_{Cl,swell}$ at progressively depolarized membrane potentials is explained by the plugging mechanism, in which negatively charged EDTA is driven into the channel's outer vestibule due to increasing driving force, where it can interact with a positively charged locus responsible for anion selectivity, thereby occluding the conducting pore for the passage of Cl^-. If this hypothesis is correct then the EDTA-induced blockade of $I_{Cl,swell}$ should be pH-dependent, increasing at alkaline pH due to an elevated proportion of the EDTA forms with higher negative charge and vice versa.

Fig. 2C shows that increasing pH of the hypo-TEA solution to 8.2 by itself produced an almost twofold enhancement of $I_{Cl,swell}$; however, subsequent exposure of the cell to 10 mM EDTA-supplemented DVC-free/hypo-TEA at this pH inhibited the current by as much as $83.4 \pm 7\%$ (at +100 mV), which was notably higher than the $49.7 \pm 6.3\%$ observed at normal pH (Fig. 2A). The reduction of the pH to 6 produced opposite effects: first, $I_{Cl,swell}$ was suppressed just by transition into hypo-TEA with pH 6, but subsequent application of 10 mM EDTA at the same pH caused only $30.7 \pm 4\%$ of current inhibition (Fig. 2D). It should be mentioned that at both pHs free Ca^{2+} concentration stayed below physiologically relevant levels (i.e. 1.3×10^{-10} M at pH 6 and 2.9×10^{-13} M at pH 8.2 assuming 10^{-6} M of residual Ca^{2+} in nominally Ca^{2+}-free solution) ruling out Ca^{2+} as possible active matter in the described effects.

Thorough inspection of the original $I_{Cl,swell}$ recordings also suggests that at pH 6 and pH 8.2 EDTA induces stronger blockade at negative voltages (Fig. 2C,D) compared to normal pH (Fig. 1B). We explain this by the fact that extracellular pH per se is capable of exerting complex effects on VRAC, which may include modulation of the interaction with the chelators. Examining the pH effects was beyond the scope of the present study, however, our preliminary observations as well as observations of others [13] suggest that pH influences the conductance and the open probability of volume-regulated channels.

3.3. Inhibition of $I_{Cl,swell}$ by Ca^{2+} chelating agents is not cell-specific

After discovering the inhibitory action of Ca^{2+} chelating agents on $I_{Cl,swell}$ in LNCaP cells we were interested to determine how universal this effect is with respect to various types of ion channels as well as with respect to $I_{Cl,swell}$ in different cell types. Since our data show that BAPTA is the most effective inhibitor 0.5 mM BAPTA in DVC-free extracellular solutions was used in this series of experiments. Apart from LNCaP cells, which in addition to $I_{Cl,swell}$ possess a voltage-gated TEA-sensitive K^+ current [10], we also used rat basophilic leukemia (RBL-2H3) cells stably transfected with the Kv1.3 K^+ channel (kindly provided by Dr. S. Grissmer, University of Ulm, Germany) and also possessing endogenous $I_{Cl,swell}$ [14], and HACaT keratinocytes, which according to our preliminary observations possess robust $I_{Cl,swell}$ as well.

Our experiments showed that neither K^+ current in LNCaP cells nor Kv1.3-mediated K^+ current in RBL cells was sensitive to 0.5 mM extracellular BAPTA. In contrast, 0.5 mM BAPTA inhibited $I_{Cl,swell}$ in RBL-2H3 cells by $49.7 \pm 5.3\%$ ($n = 6$) and $I_{Cl,swell}$ in HACaT cells by $35.7 \pm 6.4\%$ ($n = 4$). These experiments strongly suggest that sensitivity to Ca^{2+} chelating agents is likely to be a common feature of all volume-regulated anion channels irrespective of their phenotypic representation.

4. Discussion

Ca^{2+} chelating agents are widely used in biological research for Ca^{2+} buffering purposes, and their actions on cellular processes have usually been considered as being mediated only via changing free concentrations of polyvalent cations. However, there are indications that this may not always be the case, especially with respect to the relatively novel chelator BAPTA, which gradually replaces other chelators in most applications due to its higher speed of Ca^{2+} binding and better pH sensitivity [15]. There is evidence, for instance, that BAPTA (or its membrane-permeable form BAPTA-AM) and EGTA are able to directly influence Ca^{2+}-activated K^+ channels [16] and Ca^{2+} channels [17,18] in adrenal chromaffin cells and also to inhibit K^+ currents in cerebellar granule neurons [17,18] suggesting that some channels may have specific interaction sites with Ca^{2+} chelating agents.

Our study clearly establishes that various Ca^{2+} chelating agents exert differential blocking action on volume-regulated anion channels underlying $I_{Cl,swell}$. Of the three agents tested – BAPTA, EDTA and HEDTA – BAPTA appeared to be the most effective $I_{Cl,swell}$ blocker. $I_{Cl,swell}$ blockade by all agents was the strongest at positive voltages when current has an outward direction associated with inward flux of negatively charged Cl^-.

Our results on pH dependence of EDTA-induced blockade of $I_{Cl,swell}$ strongly favor the idea that the efficacy of the blockade is related to the net negative charge of the free chelator forms in the solution. This would imply the blocking mechanism, in which the negatively charged extracellular chelator molecule at membrane potentials that increase the driving force for its movement in the inward direction would enter the outer vestibule of VRAC, but because of steric limitation and/or strong binding would not be able to pass through the channel plugging it for the passage of Cl^- ions. The constancy of the $I_{Cl,swell}$ reversal potential in the absence and in the presence of the chelators suggests against their permeation through the channel. In this case the increasing of negative charge of the chelator molecule would on the one hand made it more competitive with Cl^- for the entry into the channel's vestibule and on the other hand increase its interaction with positively charged site(s) of the VRAC channel that underlie its anion selectivity (e.g. see [19]). Consistent with such blocking mechanism, $I_{Cl,swell}$ inhibition by extracellular EDTA in-

creases at more alkaline pHs, when the proportion of free EDTA forms with higher negative charge goes up. Also consistent with this mechanism is the notably higher potency of BAPTA in blocking $I_{Cl,swell}$ compared to EDTA. Indeed, BAPTA exhibits much weaker pH sensitivity, and most of it is represented by the highest negatively charged $BAPTA^{4-}$ form at all pHs above 6 [15].

Finally, our results show that $I_{Cl,swell}$ blockade by Ca^{2+} chelating agents is not specific to LNCaP cells, but represents a general phenomenon that is not dependent on the cell type. Given the possible mechanism of such blockade, we also hypothesize that other types of anion channels may be subjected to the inhibitory action of Ca^{2+} chelating agents as well. Therefore, careful selection of the type and concentration of Ca^{2+} chelators to meet specific experimental needs should be exercised.

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