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Advances in the understanding of transmembrane ionic gradients and permeabilities in smooth muscle obtained by using ion-selective micro-electrodes

by C.C. Aickin and A.F. Brading

University Department of Pharmacology, South Parks Road, Oxford OX1 3QT (England)

Key words. Smooth muscle; ions.

Introduction

Classical techniques of ion analysis and radioisotope flux have indicated a basic similarity of the transmembrane ionic gradients in smooth muscle cells to those found in the much studied preparation at the other end of the cell size spectrum, the squid giant axon. Intracellular K^+ would appear to be high and intracellular Na^+ relatively low while intracellular Cl^- seemed to be considerably higher than that predicted from a passive distribution, that is from the membrane potential (E_m) and extracellular Cl^-[21,41]. However, theoretical determination of E_m from estimates of the intracellular concentrations and resting permeabilities using the constant field equation did not yield a value close to that measured by microelectrodes, but one some 30 mV too low[18]. This inequality led to the postulation of a significant contribution to E_m from electrogenic mechanisms, for example the Na^+-K^+ pump. However, it is clear that the accuracy of the theoretical determination is critically dependent upon the accuracy of the measurements of the ionic gradients and permeabilities. Brading[13] showed that, with a different interpretation of efflux data which yielded a notably lower estimate of both intracellular Na^+ and Na^+ permeability, E_m determined by the constant field equation could be close to that directly measured in both normal and hypertonic solution. Nevertheless, it would be unwise to use this agreement as grounds for the validity of these indirect determinations.

The development of intracellular ion-selective microelectrodes clearly provided the method for reliable measurements since not only is the intracellular *activity*

rather than total concentration directly recorded, but also problems relating to compartmentalization are avoided. Yet initially the use of this technique was limited to large and robust cells like crab muscle and squid giant axon[16, 17, 32] because of the difficulty of reducing the electrode's tip dimensions. Different methods of electrode construction evolved and in 1977, Yamaguchi and Stephens appeared to have overcome the problem sufficiently for measurement of intracellular pH in smooth muscle. However, only preliminary reports were published[54, 55] and other scientists failed to reproduce the electrode[42]. In 1980 we succeeded in directly measuring the intracellular Cl^- activity in smooth muscle of the guinea pig vas deferens using independent Cl^--sensitive and voltage electrodes[3] and in the following year developed a more reliable double-barrelled micro-electrode[1]. Based on the use of liquid ion exchangers, this electrode has opened the field for investigation of virtually all physiologically important inorganic ions in smooth muscle.

1) Intracellular chloride: Methodology, steady state values and passive permeability

The major drawback of the use of any indirect method for intracellular determination, for example ion analysis, radioactive flux and electron probe analysis, is that none can distinguish between free and bound ions. Thus, as in the well known example of calcium, these methods can result in gross overestimation of the intracellular activity. Our initial measurements with Cl^--sensitive micro-electrodes were undertaken with the intention of establishing whether the presence of bound and/or sequestered ions could account for the high levels of chloride consistently found in smooth muscle (for reviews, see Casteels[21] and Prosser[41]). As soon as the first single-barrelled, Cl^--sensitive micro-electrode was successfully inserted into a smooth muscle cell, it was clear that intracellular chloride was not governed by the Donnan equilibrium but indeed was at a considerably higher activity. When an ion-sensitive electrode is inserted into a cell, it records the algebraic sum of E_m and a potential proportional to the intracellular activity of the ion. Thus if the ion was in equilibrium (and the electrode had a theoretical response to changes in ion activity) no potential difference would be recorded across the cell membrane since the equilibrium potential of the ion would equal E_m. When the Cl^--sensitive electrode was inserted into a cell of the guinea pig vas deferens, a markedly positive potential was recorded. The mean stabilized value was $+45.3 \pm 6.3$ mV (SD of an observation, $n = 44$)[3].

Surprisingly, in the light of the known degree of electrical coupling in this preparation[15, 50], cells in close proximity, simultaneously impaled with identical conventional micro-electrodes did not have the same E_m and values could vary by up to 20 mV. It is possible that this disparity results from a partial uncoupling of damaged cells but whatever the reason, its occurrence can cause a considerable error in the determination of intracellular chloride activity (a^i_{Cl}) from simultaneous, but independent impalements with Cl^--sensitive and voltage micro-electrodes. Nevertheless, in these early experiments we obtained a mean value for a^i_{Cl} of 45 mM at an E_m of -66.4 ± 5.0 mV ($n = 46$)[3], when a passive distribution would predict a value of about 8 mM.

The uncertainty about the equality of E_m in different cells can be avoided by the use of a double-barrelled electrode whereby both voltage and ion-sensitive elements are inserted into the same cell. This type of electrode is much more difficult both to manufacture and to insert successfully into a smooth muscle cell, but its advantage is enormous. Measurements with the double-barrelled micro-electrode confirmed the values obtained in normal Krebs solution with independent electrodes with a mean a^i_{Cl} of 41.2 ± 6.7 mM ($n = 79$) at an E_m of -67.6 ± 7.8 mV[4] and also demonstrated that a high a^i_{Cl} is not restricted to vas deferens. A mean value of 51.1 ± 40 mM at an E_m of -48.7 ± 5.4 mV ($n = 13$) was recorded in the guinea pig ureter[9].

We had concurrently measured intracellular chloride in guinea pig vas deferens under the same conditions as used for the electrode study with both ion analysis and back extrapolation of ^{36}Cl efflux. Values of about 50 mmole/liter cell H_2O were obtained, equivalent to an activity of about 39 mM (assuming the same activity coefficient intracellularly as determined in the normal Krebs solution). This remarkable agreement between the methods was found for all steady state values of a^i_{Cl} measured in different levels of extracellular Cl^- (Cl^-_0) as shown in figure 1. It suggests both that Cl^- ions are not bound to a significant extent and that the compartment seen by the micro-electrode is probably representative of the whole cell. Figure 1 also shows that even at very low levels of Cl^-_0, a^i_{Cl} is considerably higher than that predicted by a passive distribution – so much higher in fact that a^i_{Cl} *exceeds* the extracellular activity until this is raised to about 25 mM (i.e. an external concentration of about 33 mM).

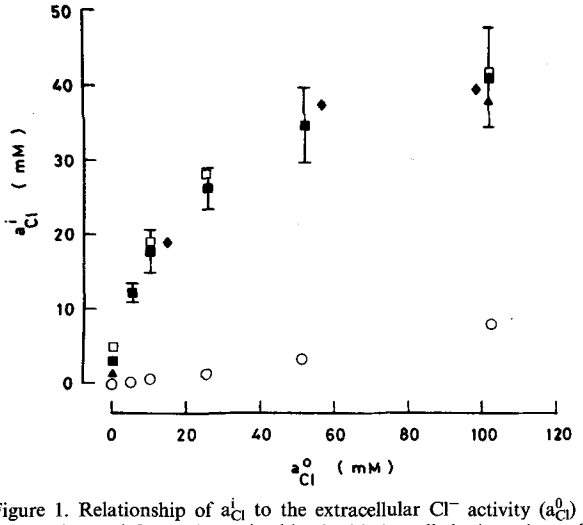

Figure 1. Relationship of a^i_{Cl} to the extracellular Cl^- activity (a^0_{Cl}) in guinea pig vas deferens determined by double-barrelled micro-electrodes (filled squares), single-barrelled micro-electrodes (open squares), ion analysis (filled triangles) and ^{36}Cl efflux (filled diamonds) compared with the relationship predicted by a passive distribution at the membrane potentials recorded in the double-barrelled micro-electrode study (open circles). Values obtained by the indirect methods were converted into an activity by multiplication with the activity coefficient determined in normal Krebs solution. The bars denote the standard deviation of the values obtained with double-barrelled electrodes, that in Cl^--free solution being within the size of the symbol.

The steady state values shown in figure 1 indicate that removal of Cl_0^- will cause a substantial fall in a^i_{Cl}. Continuous recording with a double-barrelled Cl^--sensitive micro-electrode demonstrates just how rapidly this occurs (see fig. 6 of Aickin and Brading[4] and fig. 1 of Aickin and Brading[5]). The mean time constant for the fall is 4.7 ± 0.2 min (n = 16). Such rapid movement of Cl^- ions had been observed earlier in efflux experiments[13,19] and was assumed to occur through a passive leak, thus indicating a relatively high Cl^- permeability (P_{Cl}). However, if this were the case, a large transient depolarization would occur, as in frog skeletal muscle[34], but this was not observed. Similarly, changes in E_m would affect a^i_{Cl} and this was not observed[5,9]. Furthermore, if P_{Cl} was as high as suggested (approximately equal to the potassium permeability, P_K[19,20]) membrane resistance, and hence the amplitude of the hyperpolarization caused by re-activation of the electrogenic Na pump, should increase considerably in Cl^--free solution. But this, too, was not observed[5]. Finally, we have found that cellular Cl^-, measured by ion analysis, is unaltered by prolonged and severe impairment of the Cl^- accumulating mechanism (4 h exposure to 130 µM DIDS[6]). Apart from these strong indications for a relatively low P_{Cl}, it seems intuitively unlikely that both a high P_{Cl} and a^i_{Cl} would occur in the same cell.

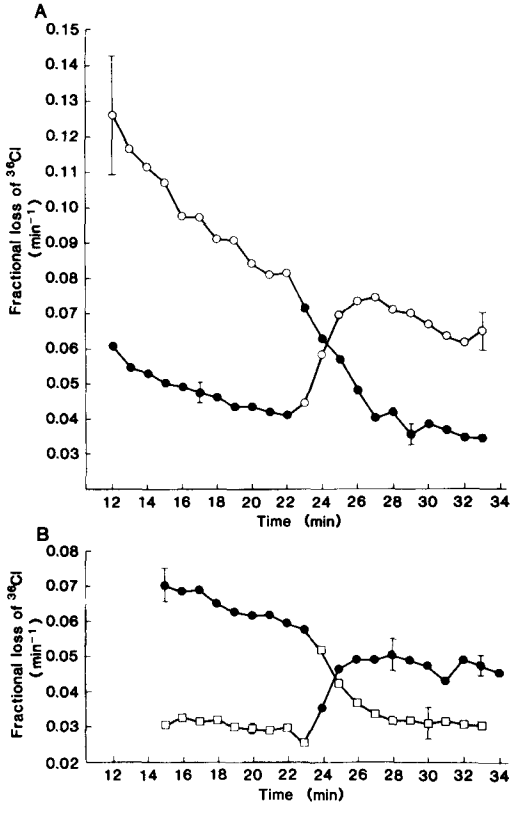

Figure 2. *A*, the effect of changing between Cl^--free (filled circles) and Cl^--containing (open circles) solution in the presence of CO_2/HCO_3^- on the instantaneous rate of ^{36}Cl efflux in guinea pig vas deferens. n = 6 for each curve. *B*, the effect of changing between nominally CO_2/HCO_3^--free (open squares) and CO_2/HCO_3^--containing (filled circles) solution on the instantaneous rate of ^{36}Cl efflux in Cl^--free conditions in guinea pig vas deferens. n = 4 for each curve. The bars denote representative standard errors in both parts of the figure.

Probably the most reliable estimate of P_{Cl} is obtained from the ratio of P_{Cl}/P_K, derived from the instantaneous changes in E_m recorded on rapid changes in external Cl^- and K^+[45], for which we have found a value of 0.044[5]. This is equivalent to a P_{Cl} of 3.9×10^{-9} cm s^{-1}, taking Casteels'[19] value for P_K of 8.9×10^{-9} cm s^{-1} in the same preparation (compared with Casteels' value for P_{Cl} of 9.7×10^{-8} cm s^{-1}). It is notable that a similarly low value (3×10^{-9} cm s^{-1}) has also been concluded from measurements of E_m in the smooth muscle of guinea pig submucosal arterioles[33]. If P_{Cl} is so low, how then can we explain the rapid efflux of ^{36}Cl in steady state conditions or indeed the rapid loss of intracellular Cl^- on removal of Cl_0^-? Clues to the explanation come from the initially puzzling observation that removal of Cl_0^- decreases the fractional loss of ^{36}Cl while readmission of Cl_0^- causes a marked stimulation, as shown in figure 2A (see also Aickin and Brading[5]), and from the observation that procedures which slow the reaccumulation of Cl^- ions against their electrochemical gradient also slow the loss of Cl^- ions when Cl_0^- is removed (Aickin and Brading[6]; see also fig. 2B). The latter observation suggests that the Cl^- accumulating mechanism is reversible and underlies both increases and decreases in intracellular Cl^- when Cl_0^- is altered, while the former suggests that in the steady state, this mechanism operates in a Cl^- self-exchange mode. Evidence for this is provided by the findings that DIDS, an irreversible inhibitor of anion exchange, considerably slows the fractional loss of ^{36}Cl in both the steady state and into Cl^--free solution while the nominal absence of HCO_3^-, a substrate of anion exchange, only slows the fractional loss into Cl^--free solution (Aickin and Brading[5,6]; see also fig. 2B). Thus we conclude that most of the transmembrane Cl^- movements are carrier-mediated as an electrically-silent exchange. As a result, estimation of P_{Cl} from the loss of intracellular Cl^- into Cl^--containing, or Cl^--free solution is liable to considerable error.

2) Mechanism for the regulation of a^i_{Cl}

The presence of a reversible Cl^- transport system was invoked in the first section for the reconciliation of rapid transmembrane movements of Cl^- ions yet very low P_{Cl} and some of its properties were given as evidence. A Cl^- accumulating mechanism is obviously required to account for the high a^i_{Cl} and its operation is demonstrated by the rapid increase in a^i_{Cl} well above the level predicted by the Donnan equilibrium when Cl^- is readmitted to the solution superfusing Cl^--depleted cells of guinea pig vas deferens, as shown in fig. 3A (see also Aickin and Brading[4-6]). Operation of this mechanism for net movement of Cl^- ions in either direction is dependent upon the presence of CO_2 and HCO_3^-: accumulation and loss of intracellular Cl^- ions (measured either directly with Cl^--sensitive micro-electrodes or by ^{36}Cl fluxes) when Cl^- is readmitted to, or removed from, the external solution respectively are slowed by their nominal absence and accelerated by their reapplication (see figs 2B and ref. 6). These observations are most simply explained by the coupling of Cl^- transport to the counter movement of HCO_3^- ions. Pronounced slowing of both the accumulation and loss of Cl^- ions by the presence of DIDS, an inhibitor of Cl^--

HCO_3^- exchange in the erythrocyte, supports this interpretation (see fig. 3A). But perhaps the strongest evidence for Cl^--HCO_3^- exchange is provided by the dependence of intracellular pH (pH_i) on Cl_0^-. Removal of Cl_0^- in the presence of CO_2 and HCO_3^- causes a rapid intracellular alkalinization (accumulation of HCO_3^- ions) which is maintained until Cl^- is readmitted to the superfusing solution when there is a similarly rapid intracellular acidification (loss of HCO_3^- ions; see fig 3B and ref. 6).

Nevertheless, Cl^--HCO_3^- exchange cannot fully account for the high a^i_{Cl} nor for net Cl^- movements when Cl_0^- is altered. Quantitative comparison of the mean changes in a^i_{Cl} and intracellular HCO_3^- activity (derived from pH_i with the assumption that the HCO_3^- activity coefficient is the same as that for Cl^-) reveals that about 25% fewer HCO_3^- ions are lost than Cl^- ions are accumulated when Cl_0^- is readmitted to Cl^--depleted cells and similarly about 25% fewer HCO_3^- ions are accumulated than Cl^- ions are lost when Cl_0^- is removed. Furthermore, the HCO_3^- movement is accomplished 2–3 times faster than the simultaneous, opposite Cl^- movement[6]. And finally, application of DIDS completely inhibits this Cl^--HCO_3^- exchange as it does in the erythrocyte, demonstrated by the total abolition of pH_i changes on alteration of Cl_0^- (fig. 3B), but Cl^- ions are still slowly accumulated and lost (fig. 3A). Thus another, HCO_3^--independent process must be involved, responsible for about 25% of net Cl^- transport when Cl_0^- is altered and probably solely responsible for accumulation of about the last 6 mM-a^i_{Cl}[6]. Inhibition of this second process may have little effect on the initial rate of transmembrane Cl^- movements but decrease the steady state a^i_{Cl}. Our preliminary experiments in guinea pig vas deferens suggest that Na^+ ions may be involved, perhaps in a Na^+-Cl^- co-transport system, since complete removal of Na_0^+ had little effect on the rate of either Cl^- accumulation or loss but lowered the steady state cellular Cl^- content. Application of the diuretic frusemide, a known inhibitor of co-transport (for review see Ellory et al.[26]), slowed both accumulation and loss of Cl^- ions, at least in part through its inhibitory action on Cl^--HCO_3^- exchange demonstrated by the slowing of pH_i changes observed on alteration of Cl_0^- (see also Lambert and Lowe[39]). But it also enhanced the inhibition caused by the presence of DIDS and decreased the steady state Cl^- content.

In concluding this section, it is notable that very similar results have been obtained using Cl^--sensitive micro-electrodes in smooth muscle from guinea pig ureter. Both reaccumulation and loss of Cl^- ions are slowed by the nominal absence of CO_2 and HCO_3^- and accelerated by their readmission, and both are substantially inhibited by the presence of DIDS[9]. Nevertheless, the major contribution of Cl^--HCO_3^- exchange to regulation of a^i_{Cl} may not be a common feature of smooth muscle. Kreye and Gerstheimer[38] have shown that steady state uptake and efflux of ^{36}Cl in rabbit aorta are enhanced by withdrawal

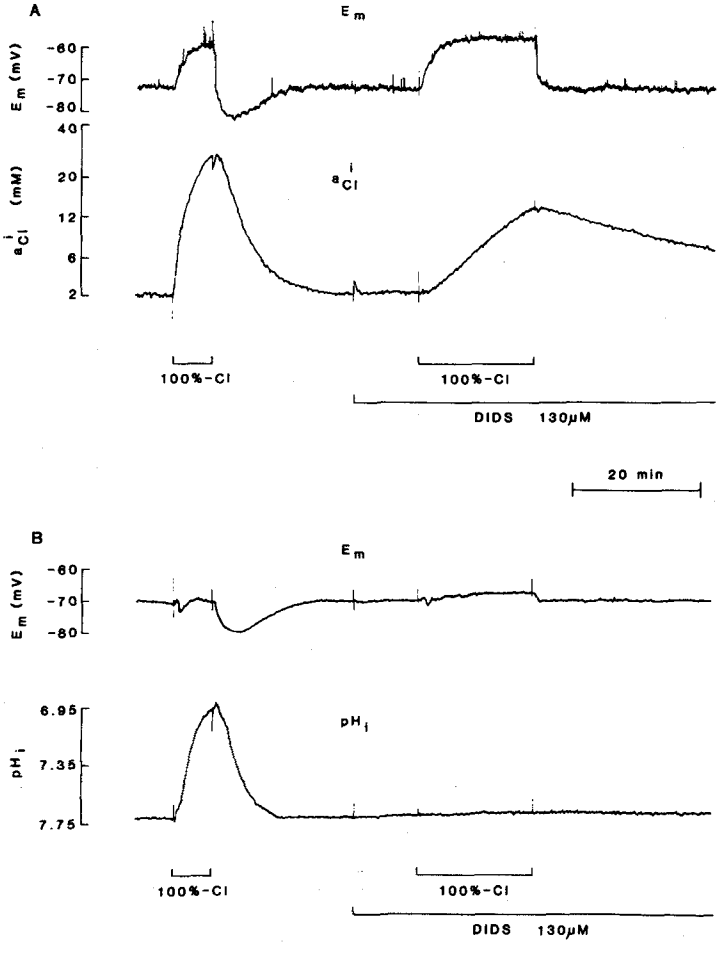

Figure 3. *A*, the effect of application of DIDS (130 μM) on the rate of reaccumulation and loss of a^i_{Cl} in guinea pig vas deferens. The preparation was superfused with Cl^--free solution except for the periods indicated and all solutions were equilibrated with 3% CO_2/97% O_2 at pH 7.35. *B*, the same protocol repeated during measurement of pH_i.

of CO_2 and HCO_3^- whereas efflux into Cl^--free solution is unaffected. In addition, Cl^- efflux is not significantly affected by the presence of DIDS (V.A.W. Kreye, personal communication). However, frusemide decreases both ^{36}Cl efflux into Cl^--containing solution and total Cl^- content in this preparation[37]. Thus it is possible that a HCO_3^--independent, frusemide-sensitive process may be common to the regulation of a^i_{Cl} in smooth muscle.

3) Intracellular pH: Methodology, steady state values and the effect of CO_2

Despite the number of indirect methods available for estimation of pH_i, surprisingly few measurements have been made in smooth muscle[2]. Those that have, suggest that the normal value is not significantly different from that found in many other preparations, with the exception of the early work of Furusawa and Kerridge[30] who found a considerably more alkaine value for cat uterus homogenates than for gastrocnemius or cardiac muscle. Their method was obviously crude, yet their values for skeletal and cardiac muscle are remarkably similar to those reported more recently by direct measurement of pH_i with pH-sensitive micro-electrodes[8,23,25,53]. As mentioned in the Introduction, Yamaguchi and Stephens[54,55] appeared to have overcome the technical problems for direct measurement in smooth muscle. They reported a value of 7.05 for dog tracheal muscle, very close to values reported for mouse soleus muscle (7.07)[8] and sheep heart Purkinje fibers (7.02 and 7.09)[25,53]. However, the irreproduceability of their design of electrode (see Roos and Boron[42]) casts doubt on the validity of their measurements.

None of the proven electrodes previously used intracellularly, which have their function conferred by the use of pH-sensitive glass, seem ideally suited, or even likely to be easily modified, for use in smooth muscle. The Thomas-style recessed-tip electrode[48] has been successfully used in the small cells of Salamander renal proximal tubule as a single-barrelled electrode[12] and has also been incorporated into a double-barrelled configuration for use in mammalian cardiac and skeletal muscle[23]. However, the double-barrelled form has a total tip diameter around 1 μm – too large for successful impalement of a smooth muscle cell. Thus the use of a liquid ion exchanger, sensitive to pH seemed to offer the greatest chance of success. Such an ion exchanger could be incorporated into the double-barrelled electrode originally perfected for measurement of a^i_{Cl}[1].

A HCO_3^--sensitive exchanger was reported in 1974 and was used by Khuri and co-workers in both frog and rat skeletal muscle[35,36]. Reasonable values of pH_i were obtained. But the exchanger has a limited application since it is apparently sensitive to changes in P_{CO_2} and, in addition, has not worked in the hands of other researchers. A nigericin-based exchanger was briefly described in 1980[40] and yielded reasonable values for pH_i of frog muscle and renal proximal tubule cells. However, it showed some sensitivity to K^+, Na^+, Ca^{2+} and Mg^{2+} and substantial sensitivity to NH_4^+. The use of another H^+-sensitive exchanger was described in 1981[31] which seemed more promising but again proved only to function, at least when incorporated into a micro-electrode, in the hands of the original investigators. Most recently, Simon and his colleagues developed a ligand which showed good selectivity for H^+ ions[10] and they reported measurements in Xenopus eggs very similar to those found previously with the Thomas electrode[51]. This ligand appears to be superior to all the forerunners and was consequentially chosen for the attempt to determine pH_i in smooth muscle. Initial experiments in mouse soleus muscle, compared with those previously made in this preparation using the Thomas electrode[8], confirmed the reliable intracellular function of this ligand in a double-barrelled electrode suitable for use in guinea pig vas deferens[2].

Successful impalement of a smooth muscle cell with this double-barrelled electrode confirmed the suspicion that pH_i is considerably more alkaline than would be expected if H^+ ions were passively distributed across the cell membrane, although not as alkaline as suggested by Furusawa and Kerridge[30]. The mean pH_i of cells from the guinea pig vas deferens in normal Krebs solution is 7.06 ± 0.09 ($n = 52$) at an E_m of -65.5 ± 6.7 mV, remarkably close to the values found under similar conditions in other mammalian muscle types: pH_i predicted by a passive distribution would have been 6.28[2]. It is worth pointing out that, at least in vas deferens, H^+ ions are therefore further from equilibrium across the smooth muscle cell membrane than are Cl^- ions under the same conditions. The mean Cl^- equilibrium potential (E_{Cl}) in normal Krebs solution is -24.3 mV[4] while that for H^+ ions (E_H) is -17.1 mV.

As already described for Cl^-, some idea of the stability of this disequilibrium can be gained from alteration of the extracellular level. However, the effect of alteration of extracellular pH (pH_o) on pH_i is more complex than that of Cl^-_0 on a^i_{Cl} because, in the presence of CO_2, pH is dependent upon two variables; CO_2 and HCO_3^-. Variation of pH_o at constant CO_2 (by variation of HCO_3^-) causes a relatively slow change in pH_i (see fig. 4). The change in pH_i is about 40% of that in pH_0 and is complete in 6–12 min[2]. This is considerably faster than observed in either mammalian skeletal or cardiac muscle[8,25], presumably reflecting the larger surface area to volume ratio of these small cells. If the same change in pH_0 is made by alteration of both CO_2 and HCO_3^-, the change in pH_i is completed much more rapidly (in about 2 min; see fig. 4). This difference has been well documented in other preparations and can be explained by the rapid diffusion of CO_2 but not of H^+, OH^- or HCO_3^- ions across the cell membrane. One might be tempted to estimate P_H from the acidification observed in constant CO_2 but it seems likely that, as in the case of Cl^-, this change in pH_i is carrier-mediated[27,43,49]. Indeed for this change to be caused by a passive leak, P_H would have to be of the order of 3×10^{-1} cm s^{-1} 2!

Thus far pH_i of smooth muscle behaves in a similar fashion to that of other mammalian muscle types, but it exhibits greater stability when CO_2 is altered. Increasing or decreasing CO_2 at constant pH_o results in the expected, rapid changes in the pH_i of smooth muscle as CO_2 reequilibrates across the cell membrane. pH_i then returns to the previous steady state value. Neither skeletal nor cardiac muscle are capable of completely restoring pH_i under these conditions and thus their pH_i is more acidic at higher CO_2; more alkaline at lower CO_2[8,25]. When CO_2 is altered at constant HCO_3^- (alteration of pH_0), the same

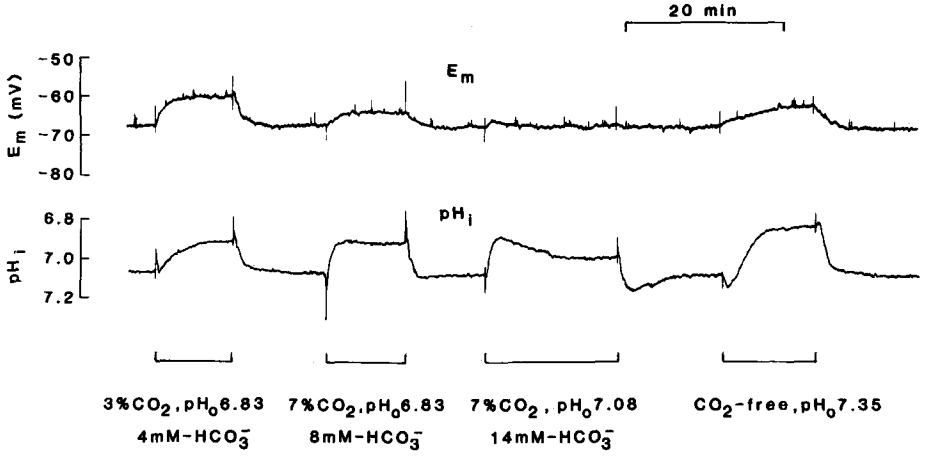

Figure 4. The effect of alteration of CO_2 and external pH on pH_i in guinea pig vas deferens. The preparation was superfused with normal Krebs solution containing 14 mM-HCO_3^- and equilibrated with 3% CO_2 at pH 7.35 except for the intervals indicated. CO_2-free solution was buffered with HEPES and equilibrated with 100% O_2.

initial rapid changes in pH_i occur but then pH_i of smooth muscle partially recovers (see fig. 4), i.e. equivalent to the behavior of other muscle types when CO_2 is altered at constant pH_0. The same procedure in skeletal and cardiac muscle results in complete failure to recover from the initial change.

It appears that this greater stability of smooth muscle pH_i is CO_2/HCO_3^- dependent since it is lost in their nominal absence. Removal of CO_2 results, in the steady state, in a completely unexpected acidification, pH_i stabilizing at a value at least 0.2 units more acid than in the presence of CO_2. The mean pH_i in the nominal absence of CO_2 and HCO_3^- is 6.81 ± 0.11 ($n = 18$) with an E_m of -60.8 ± 8.2 mV[2]. As indicated above, pH_i of both skeletal and cardiac muscle stabilizes at a more alkaline level. This acidification in smooth muscle can be so pronounced that the expected, initial alkalinization is almost obscured (see fig. 4). Replacement of CO_2 and HCO_3^- results in a rapid alkalinization which in some cases is so rapid that the predictable acidification is obscured (see fig. 4). pH_i then returns to its previous value in CO_2-containing solutions in about 10 min. This unique behavior requires further investigation for its cause to be elucidated but, if common to all types of smooth muscle, may have important repercussions for the interpretation of vascular responses to changes in CO_2 and pH.

Clearly this close regulation of pH_i at a level so far from equilibrium requires the presence of a mechanism for actively extruding H^+, or alternatively for accumulating OH^- or HCO_3^- ions. To date, we know little about its characteristics. However, it does appear that a CO_2/HCO_3^--dependent process confers a powerful part of the cells' ability to extrude H^+ ions. This process does not require the presence of Cl^- ions, either intra- or extracellularly, although Cl^- ions do have a significant effect on pH_i. Another process is probably also present since pH_i remains about 0.6 units more alkaline than predicted by the Donnan equilibrium in the nominal absence of CO_2 and HCO_3^-. Finally, operation of this mechanism does not cause marked changes in E_m, suggesting that it is electroneutral. These observations indicate that the mechanism is unlike any so far described for the regulation of pH_i (for review see Roos and Boron[42], and a subsequent mechanism, Boron and Boulpaep[12]) but a complete investigation is required to determine its properties.

4) Intracellular sodium: Methodology, steady state values and passive permeability

The use of indirect methods for estimation of intracellular sodium suffers from the same drawbacks as it does for chloride. The problems are, if anything, accentuated in the case of sodium, first because intracellular binding occurs[24,29] and secondly because rapid transmembrane movement of Na^+ ions in smooth muscle compromises the required washout of extracellular Na^+[13]. Unfortunately, limited experience with a Na^+-sensitive microelectrode suitable for impalement of smooth muscle cells suggests that this electrode is the most capricious of the ion-selective electrodes used so far.

Undoubtedly, the best Na^+-sensitive electrode is the recessed-tip variety whose function is conferred by Na^+-sensitive glass[47]. But this design, particularly when encorporated into a double-barrelled electrode[56], poses real problems for manufacture in dimensions feasible for impalement of smooth muscle. The technically easier solution of employing the Na^+-sensitive liquid ion exchanger (ETH 227)[44] in the double-barrelled micro-pipette previously used for measurement of a^i_{Cl} and pH_i has therefore been adopted at the expense of poorer selectivity. This exchanger shows interference from Ca^{2+} as well as K^+ and the interference increases with decreasing diameter of the tip of the micro-pipette in which the exchanger is placed: a phenomenon which has also been observed with the Cl^--sensitive liquid ion exchanger[4]. Not only is the interference relatively high in electrodes capable of being inserted into smooth muscle, but the relative contribution from Ca^{2+} and K^+ varies from electrode to electrode. Thus each electrode has to be characterized so that the necessary corrections can be made. Because of the Ca^{2+} sensitivity, this is done at the end of an experiment in modified Krebs solution from which Ca^{2+} has been omitted, with K^+ as the Na^+ substitute. Constancy of the electrode's sensitivity is shown by comparison of the response to a ten fold reduction of Na^+ in Krebs solution, substituted by Tris, recorded before and after the experiment. Sensitivity is usually lost when repeated attempts are required to obtain a successful impalement and is often short-lived following successful impalement. Nevertheless, when an electrode maintains its sensitivity intracellularly, experiments can be performed which last for many hours and, unlike with the Cl^-- and pH-sensi-

tive electrodes, drift rarely occurs. At present it is not known how well the calibrating solutions correspond to the sarcoplasm, particularly with respect to Ca^{2+}, and therefore the values for intracellular Na^+ activity (a^i_{Na}) should be considered as a best estimate. However, it seems unlikely that they are much in error.

Measurements have been made in the guinea pig ureter following experiments which indicated the presence of Na^+-Ca^{2+} exchange in this preparation[7]. A mean a^i_{Na} of 8.1 ± 2.9 mM (n = 21) has been recorded with an E_m of −49.5 ± 4.2 mV (Krebs solution buffered with Tris chloride to pH 7.35 at 35 °C, equilibrated with 100% O_2). Thus the Na^+ equilibrium potential (E_{Na}) was of a mean +64.3 mV. Reduction of Na^+_0 results in a rapid fall in a^i_{Na} as shown in figure 5. This fall is approximately described by a single exponential and has a half time of about 1.6 min. It should be noted that this fall occurs in the face of persistent inwardly-directed electrical and chemical gradients for Na^+. The mean a^i_{Na} recorded in 10%-Na^+_0 was 1.1 ± 0.6 mM (n = 17), equivalent to an E_{Na} of +56.2 mV, while E_m was −49.4 ± 5.0 mV. Replacement of normal Na^+_0 results in a rapid increase in a^i_{Na} to the previous level. This, too, can be approximately described by a single exponential and has a half time of about 0.8 min. Similar results have been observed in both crab muscle fibers[52] and mammalian cardiac muscle[22,24] and Na^+–Ca^{2+} exchange has tentatively been suggested to be involved. Under normal conditions, however, no significant change in tension is observed on complete removal of Na^+_0 in this preparation[7] and, as pointed out previously[24], these changes in a^i_{Na} could simply reflect changes in the passive influx of Na^+ ions and the activity of the Na^+–K^+ pump.

If the Na^+–K^+ pump was the only mechanism responsible for the maintenance of a low a^i_{Na}, its inhibition would result in a continual run down of the transmembrane Na^+ and K^+ gradients until eventually the cell membrane was disrupted osmotically. However, inhibition results in a slow, but restricted rise of a^i_{Na}. Stabilization occurs after 20–30 min in the presence of ouabain (10^{-4} M) and 45–60 min in the absence of external K^+, as illustrated in figure 5. The slower stabilization observed on removal of extracellular K^+ probably results from leakage of intracellular K^+ maintaining a very low level of Na^+–K^+ pump activity[7]. Most of these experiments were performed in lowered Na^+_0 (at an activity of 70 mM instead of the normal 92 mM) and the mean stabilized a^i_{Na} was 18.1 ± 2.3 mM (n = 5), equivalent to an E_{Na} of +36 mV. In addition, contrary to the expected run down, E_m became more negative, reaching a value of −64.0 ± 2.7 mV (n = 5) in the absence of external K^+ and −60.3 ± 2.1 mV (n = 3) in the presence of ouabain[7].

Thus, as previously suggested for this preparation[7], a second mechanism capable of extruding Na^+ ions against their electrochemical gradient must be present in addition to the Na^+–K^+ pump. Many lines of evidence suggest the existence of Na^+–Ca^{2+} exchange in other preparations[46] and it is notable that the guinea pig ureter develops a Na^+-withdrawal contracture after inhibition of the Na^+–K^+ pump, either by removal of external K^+ or application of ouabain, with much the same time course as the stabilization of a^i_{Na}[7]. Significantly, despite inhibition of the Na^+-K^+ pump, reduction of Na^+_0 still results in a rapid fall in a^i_{Na} (see fig. 5), still in the face of inward electrical and chemical Na^+ gradients. This reinforces the argument for a second Na^+-extrusion mechanism since without it, reduced passive Na^+ entry could only result in a decreased rate of rise of a^i_{Na}. Preliminary experiments have shown considerable slowing of the fall in a^i_{Na} on reduction of Na^+_0 by the presence of 10 mM–Mn^{2+} when the Na^+–K^+ pump is inhibited. This supports the suggestion of Na^+–Ca^{2+} exchange involvement[11] and is consistent with the observation of substantial inhibition of the Na^+-withdrawal contracture under the same conditions[7].

These initial observations with direct and continuous measurement of a^i_{Na} in guinea pig ureter confirm beyond doubt a very low normal level and consequently very

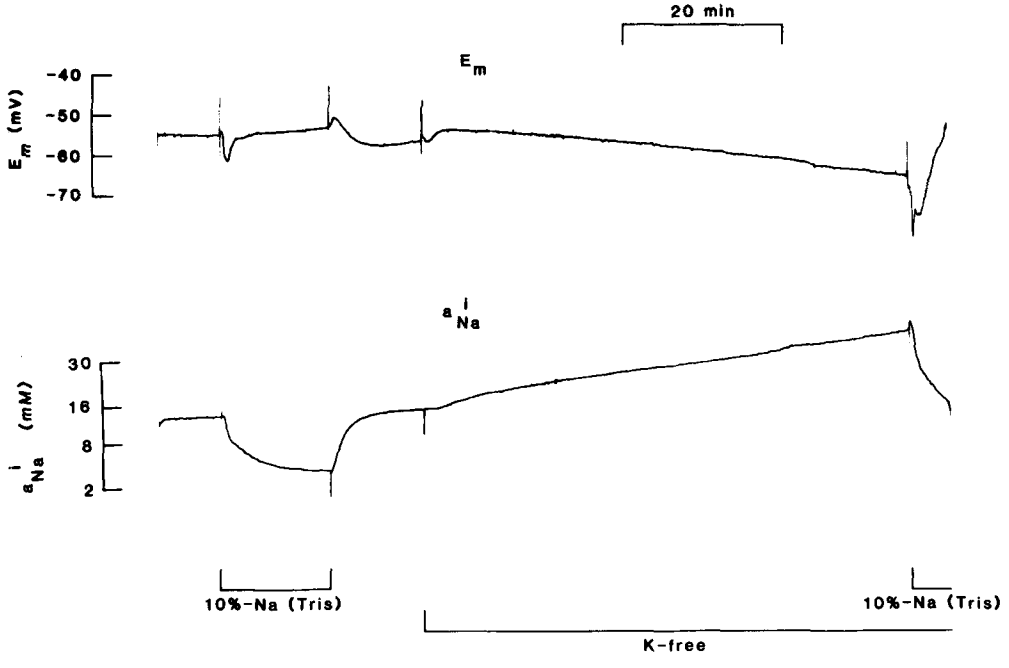

Figure 5. The effect of reduction of external Na^+ on a^i_{Na} in the presence and absence of a functional Na^+-K^+ pump in guinea pig ureter. The Na^+-K^+ pump was inhibited by removal of external K^+. All solutions were buffered with Tris to pH 7.35 and equilibrated with 100% O_2. Unfortunately the electrode was dislodged during the second exposure to 10%-Na^+, presumably due to the development of the Na^+-withdrawal contracture.

positive value for E_{Na}. They illustrate a rapid effect of Na_0^+ on a_{Na}^i, contrary to the very slow equilibration concluded from indirect measurements[28], and unequivocally demonstrate the presence of a mechanism other than the Na^+-K^+ pump for extruding Na^+ ions against their electrochemical gradient. The presence of carrier-mediated Na^+ movements throws doubt on the validity of estimates of Na^+ permeability (P_{Na}) from any transmembrane Na^+ flux or from changes in a_{Na}^i as discussed in the first section with respect to estimates of P_{Cl} (see also Aickin and Brading[5]). The rate of rise of a_{Na}^i on restoration of normal Na_0^+ in the presence of a functional Na^+-K^+ pump would suggest a P_{Na} of 1.9×10^{-7} cm s^{-1} if passive influx of Na^+ ions was solely responsible. Similarly the rate of rise of a_{Na}^i on inhibition of the Na^+-K^+ pump by ouabain would suggest a P_{Na} of 6.9×10^{-8} cm s^{-1} (for method of calculation see Aickin and Brading[5], Brading[13]). Both are in the same order or higher than usually quoted for P_K[14] and are therefore probably overestimates.

Conclusion

The ability to apply ion-selective electrodes intracellularly in smooth muscle has provided a major advance in our understanding of the distribution of inorganic ions and of their movements across the cell membrane. Direct measurement of activity, the physicochemically important parameter, has revealed for example that virtually all cellular Cl^- is uncomplexed while perhaps as much as two thirds of the cellular Na^+ is bound or sequestered. As a result, estimates of equilibrium potentials from indirect methods (e.g. ion analysis, radioisotope flux and electron probe analysis) are fortuitously accurate for Cl^- but they are considerably too negative for Na^+. Continuous recording afforded by ion-selective electrodes has demonstrated that surprisingly rapid changes in intracellular activity occur when extracellular levels are altered and significantly not only with extracellular levels of the same ion but also with those of different ions, for example the change in pH_i on alteration of Cl_0^-. The necessary simultaneous measurement of E_m has clearly shown that these changes in ion activity cannot be explained by passive movement through conventional ion channels. In the case of Cl^- and H^+ ions, no large change in E_m occurs and in the case of Na^+ ions, some of the movements are against both electrical and chemical gradients. These observations imply the presence of carrier-mediated ion movements and complementary flux experiments have shown that, at least for Cl^- ions, these also persist in the steady state in the self-exchange mode.

An important lesson which has thus been learned is that ion fluxes, even in the steady state, may not give a valid estimate of permeability through membrane channels. The rapidity with which the intracellular ion activity can change in response to alteration of the external medium also has important implications for the interpretation of experiments designed to investigate the underlying ionic conductances involved in the actions of drugs or transmitters. Extracellular ions are often changed in such experiments to determine whether they carry the activated current. Our experiments have shown that the time course and sometimes the direction of the change in driving force on the ion resulting from such a manipulation would have been very hard to predict. For instance, lowering the extracellular Cl^- to below a concentration of about 35 mM in vas deferens actually reverses the chemical gradient and thus results in an increased outward driving force.

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Patch and whole-cell voltage clamp of single mammalian visceral and vascular smooth muscle cells

by T. B. Bolton, R. J. Lang, T. Takewaki and C. D. Benham

Department of Pharmacology, St George's Hospital Medical School, London SW17 ORE (England)

Summary. Dispersal of the constituent cells of mammalian visceral and vascular smooth muscles has permitted recordings both of membrane currents under whole-cell voltage clamp, and of currents through single ionic channels using the patch-clamp technique. A rectangular depolarizing step applied to a single cell under voltage clamp yielded a net inward current followed by a net outward current in normal physiological solution. In isolated, 'inside-out' patches of cell membrane a calcium- and potential-sensitive K channel (100 pS conductance) and a calcium-insensitive, potential-sensitive K^+ channel (50 pS conductance) with slow kinetics have so far been identified and characterized.

Key words. smooth muscle; K channels; patch clamp.