# A review of the electrophysiological, pharmacological and single channel properties of heart ventricle muscle cells in the snail *Lymnaea stagnalis*

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*Abstract.* Although a considerable body of information has accumulated describing the pharmacological properties of a wide range of molluscan muscle types, the physiological bases underlying these properties have not been thoroughly investigated. At present, little is known about the types of ion channels and their regulation in molluscan muscle cell membranes. Voltage-clamp, and more recently, patch-clamp techniques have revealed molluscan muscles possess a complex array of channel types with various pharmacological and electrophysiological properties. The gating properties of these channels and their modulation by chemical agents, however, are still poorly understood. This review summarizes some aspects of molluscan muscle function with particular reference to the heart ventricle muscle of the pond snail, *Lymnaea stagnalis*.

*Key words.* Snail heart; electrophysiology; pharmacology; ion channels.

## *Introduction*

The molluscan nervous system continues to be a boundless source of information for neurobiologists. The large size of neuronal cell bodies and axons in molluscan species has facilitated all manner of investigations. Indeed, it may be argued that the development of modern membrane biophysics began with the investigation of the squid giant axon [27, 49–53]. The comparative simplicity of molluscan neuronal networks and the relative ease of identification of specific neurons have promoted significant contributions to our understanding of simple behavioural reflexes [5, 57, 84]. Furthermore, the accessibility and ease of manipulation of molluscan nervous systems has facilitated the application of a variety of electrophysiological techniques to the investigation of basic ionic mechanisms underlying axonal and somatic membrane permeability, e.g., voltage clamp [34, 38, 49–51, 53, 63, 68, 75], intracellular recording with glass microelectrodes [59, 76, 85], ion-sensitive electrodes [47, 86], internal perfusion of the squid axon [3], whole-cell perfusion [25, 62], and patch-clamp [81].

In sharp contrast, molluscan muscles have received relatively little attention. Perhaps this is due to the relatively small size or inaccessibility of many molluscan muscles, or to the extensive diversity of molluscan muscle types [39, 54]. Or it may simply be that, from an anthropocentric perspective, vertebrate muscles, with their inherently greater appeal, have provided suitable preparations for intracellular investigations. Whatever the reason, our knowledge of molluscan muscle electrophysiology is quite elementary.

This review does not attempt to present a comprehensive evaluation of the current state of knowledge of molluscan muscle physiology, but rather to summarize some electrophysiological, pharmacological and single channel properties of one molluscan muscle type, the heart ventricle muscle of the pond snail *Lymnaea stagnalis*. Nevertheless, to provide a comparative perspective, a brief overview of the morphology and electrophysiology of other molluscan muscle types is in order.

## *Morphology and electrophysiology of molluscan muscles*

Molluscan musculature is represented by diverse structural and functional types. The majority of the musculature is composed of small, non-striated uninucleate cells. Discrete muscle systems are often difficult to recognize and are virtually inseparable. Only shell adductors (e.g., the adductor muscles of lamellibranch molluscs) and certain retractor and protractor muscles from discrete units (e.g., byssus, radula, penis or antenna retractor and penis protractor muscle), but even these may be composed of different muscle types (e.g., the shell adductor muscle of *Pecten* [39, 70]). Those molluscan muscles which have been most extensively studied either form definable anatomical units (e.g., heart, buccal mass, penis retractor muscles) or are physiologically unique (e.g., the anterior byssus retractor muscle (ABRM) of *Mytilus* which is capable of maintaining prolonged tonic contractions with very little energy expenditure [90].

Unlike vertebrate muscle, molluscan muscle cannot be readily classified into 'smooth' and 'striated' types. Some molluscan muscles exhibit both physical and physiological properties which are intermediate between smooth and striated types, for which various names have been proposed. Heyer et al. [44] suggested a comprehensive classification scheme for invertebrate muscles in general. However, the classification scheme of Hanson and Lowy [40] and Hoyle [54] are most often used in the literature. The heart ventricle muscles of *Lymnaea stagnalis* fall into the category of 'cross-striated' (ordinary striated) muscle fibres [77] which are identical in appearance under the light microscope to vertebrate and arthropod skeletal muscle fibres. They are divided into sarcomeres by regularly spaced, well-defined 'Z-bands' and exhibit characteristics of A and I bands. The molluscan 'Z-bands', however, are not ultrastructurally similar to vertebrate Z-bands in that they are composed of discrete 'Z-particles' or 'Z-bodies' rather than continuous 'Z-lines'.

*The potassium-dependence of the resting potential in molluscan muscle*

The ionic dependence of the resting potential in molluscan muscles has been investigated in only a few species. The membrane potential ($V_m$) appears to be a function, to a greater or lesser extent, of the extracellular $K^+$ concentration ($[K^+]_o$). Hill et al.[45], applying the sucrose gap technique to the radula protractor of *Busycon canaliculatum*, reported an average resting $V_m$ of $-55$ mV. An increase in $[K^+]_o$ resulted in a depolarization of the membrane, whereas a decrease promoted a hyperpolarization. Burnstock et al.[24] reported a resting $V_m$ of $-60$ mV for *Poneroplax albida* rectal muscle and $-38$ mV for the posterior jugalis muscle (sucrose gap), although they considered that the latter value may have been artificially low. The superfusion of the rectal muscle with isotonic KCl resulted in a 44 mV depolarization of $V_m$. Twarog[89] reported an average resting $V_m$ of $-65$ mV for the ABRM of *Mytilus edulis*. The $V_m$ was depolarized by 45 mV per decade increase in $[K^+]_o$. Kater et al.[58] recorded the resting $V_m$ of the posterior jugalis and columellar muscles of *Heliosoma trivolvis* intracellularly. These averaged $-40$ mV and $-27$ mV, respectively, although the resting $V_m$ of the columellar muscles was occasionally found to be as high as $-40$ mV, suggesting the possibility of membrane damage by impalement with microelectrodes. Kidokoro et al.[60], recording intracellularly, reported a resting $V_m$ of $-42$ mV for the adductor muscle of *Anodonta implicata*. Florey and Kriebel[31] measured a resting $V_m$ of $-20$ to $-50$ mV for the chromatophore muscles of *Loligo opalescens*, and in the radula protractor of *Rapana thomasina*, the resting $V_m$ was found to be $-45$ mV[61]. The ionic basis of these resting potentials was not investigated.

Wilkens[94] reported a resting $V_m$ of $-55$ mV in the heart muscle of the bivalve *Modiolus demissus* when recording intracellularly, and $-53.8$ mV when recording with the sucrose gap technique. In this species the resting potential was found to be primarily dependent on $[K^+]_o$ but also exhibited a significant permeability to anions and $Na^+$. Depending on the anion, the slopes per decade increase in $[K^+]_o$ ranged from 34 to 50 mV. Dorsett and Evans[28], recording intracellularly, reported a resting $V_m$ of about $-74$ mV for the buccal retractor muscle of *Philine aperta*. The resting $V_m$ of this muscle was also primarily dependent on $K^+$ and varied with slopes of 50.6 mV per decade change in $[K^+]_o$ when the $[Cl^-]_o$ remained constant, 48.5 mV in chloride-free saline, and 55.8 mV when $[K^+]_o$ $[Cl^-]_o$ was constant, again suggesting a sizeable chloride permeability in the resting muscle membrane.

Shigeto[80], recording intracellularly from *Mytilus edulis* and *Crassostrea gigas* myocardia, reported a resting $V_m$ of about $-45$ mV for both species. The $V_m$ was altered by about 30 mV per decade change in $[K^+]_o$ when $[K^+]_o$ was between 30 and 186 mM. However, no change in the resting $V_m$ was observed with $[K^+]_o$ between 1 mM and 18 mM. When $Cl^-$ was replaced with sulphate, a gradual depolarization ensued. Whether this depolarization was due to a genuine contribution of $Cl^-$ ions to $V_m$ or to some effect of sulphate on membrane properties was not ascertained. If $Cl^-$ contributes to the resting $V_m$ in these muscles, it might have been expected that alterations in $[Cl^-]_o$ would have resulted in more rapid changes in $V_m$ than were observed (cf. Hodgkin and Horowicz[48]), although $Cl^-$ trapped in interstitial spaces could mask a rapid response.

Given the broad range of reported resting $V_m$ values, it would seem that there is considerable diversity in the ionic permeability of molluscan muscle cell membranes. Moreover, the resting $V_m$s of molluscan muscles are less negative compared to vertebrate skeletal muscles (cf. $-92$ mV for frog sartorius muscle, Adrian[1]). In some cases, at least, a low resting $V_m$ might reflect damage to the sarcolemma during impalement. For example, during intracellular recording from *Anodonta* obliquely striated muscle, $V_m$ declined rapidly following impalement[60], which would suggest membrane damage had occurred. The values for resting $V_m$s determined by high-resistance gap techniques, on the other hand, incorporate other errors in measurement. For example, Sugi and Yamaguchi[83], using an oil-gap voltage clamp, reported a resting $V_m$ of $-12$ to $-25$ mV for the ABRM of *Mytilus edulis*, whereas Twarog[90], recording intracellularly, reported a resting $V_m$ of $-65$ mV for the same preparation.

The equilibrium potential of $K^+$ ($E_K$) may be comparatively low in some molluscan muscles. For example, $E_K$ in myocardial muscle fibres of *Crassostrea gigas* was calculated to be $-68$ mV[80] (which is considerably less negative than the $E_K$ of about $-90$ mV in vertebrate skeletal muscles[1,48]. Our results suggest that a similar situation may also exist in *Lymnaea* heart ventricle muscle cells (see below).

*The ionic basis of the resting potential in* Lymnaea stagnalis

Heart ventricle cells of *Lymnaea stagnalis* have a resting $V_m$ of about $-60$ mV[15]. In these cells, $V_m$ was sensitive to changes in both extracellular $K^+$ and $Cl^-$ concentrations. However, $V_m$ was relatively insensitive to changes in $[K]_o$ below about 6 mM. Between 6 mM and 40 mM, regressions of the plot of $V_m$ against $[K]_o$ defined an average slope of about 50 mV per decade change in $[K]_o$ (fig. 1). The insensitivity of $V_m$ to small changes in $[K^+]_o$ and the deviation from the predicted Nernst slope of about 58 mV for a purely potassium-selective membrane led to the suggestion that these cells are also permeable to $Na^+$. However, the expected hyperpolarization upon removal of extracellular $Na^+$ (cf. Sattelle[79]) was only observed when the extracellular $Ca^{2+}$ was reduced to 0.5 mM and the extracellular $Mg^{2+}$ was elevated to 10 mM. When the $Ca^{2+}$ and $Mg^{2+}$ concentrations were

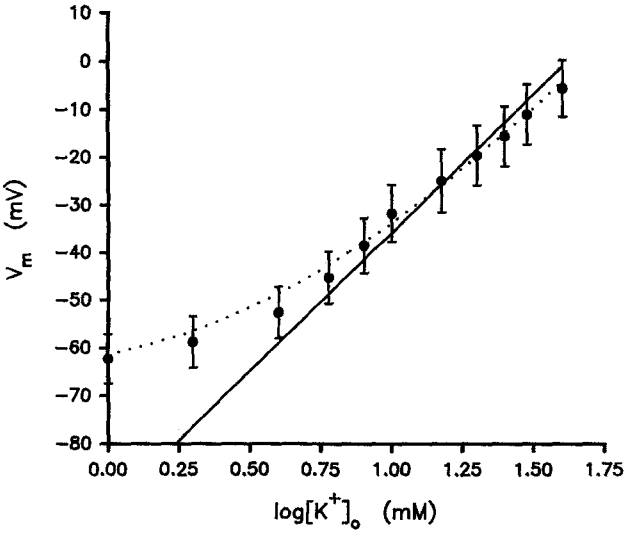


Figure 1. Relationship between the extracellular $K^+$ concentration and the membrane potential in *Lymnaea* heart ventricle muscle cells. The filled circles are the experimentally determined points. The solid line denotes the expected response for a purely potassium-selective membrane using an intracellular $K^+$ concentration of 51.5 mM. The dotted line was calculated using the modified Goldman–Hodgkin–Katz equation (see text) with an internal $K^+$ concentration of 51.5 mM, a $Na^+$:$K^+$ permeability ratio of 0.07 and an extracellular $Na^+$ concentration of 50 mM. Combined data from 20 cells were taken from Brezden and Gardner[15].

at their normal levels of 3.5 mM and 2.0 mM, respectively, the removal of sodium produced a depolarization. These results suggested that the removal of $Na^+$ from the extracellular phase inhibited, or possibly reversed, a $Na^+ - Ca^{2+}$ exchange mechanism[2], and the consequent influx of $Ca^{2+}$ was responsible for the observed depolarization. Nevertheless, the hyperpolarization observed upon the removal of extracellular $Na^+$ under appropriate conditions suggested that these cells do have a resting permeability to $Na^+$. Indeed, the data could be well-fitted to the following modified form of the Goldman–Hodgkin–Katz equation with $P_{Na}/P_K$ ratios of 0.01 to 0.14 (cf. Moreton[71]).

$$e^{VF/RT} = \frac{[K^+]_o}{[K^+]_i} + \frac{P_{Na}[Na^+]_o}{P_K[K^+]_i}.$$

The inverse slopes of regressions of plots of $e^{VF/RT}$ against $[K^+]_o$ gave a mean of about 51.5 mM for the intracellular potassium concentration ($[K^+]_i$) in *Lymnaea*[15]. This is considerably lower than the $[K^+]_i$ reported for vertebrate cardiac muscles (> 100 mM)[65, 92].

A change in $[Cl^-]_o$ from 62.6 mM to 5 mM resulted in a transient, 6 mV depolarization. The restoration of normal $[Cl^-]_o$ produced a transient hyperpolarization of about 7 mV. That these changes were considerably lower than the predicted change of 32 mV for a $Cl^-$-permeable membrane was probably due a relatively rapid re-equilibration of intracellular $Cl^-$ during the slow rate of solution exchange. In any case, the membrane potential always stabilized at a level about 2 mV more negative than the original $V_m$ following a reduction in $[Cl^-]_o$, and 1 mV more positive than the original $V_m$ following the restoration of the original $[Cl^-]_o$. In frog skeletal muscle, the rapid restoration of the original $V_m$ following changes in $[Cl^-]_o$ was attributed to an insignificant change in $[K^+]_i$[48]. However, the results with *Lymnaea* heart muscle suggested that $[K^+]_i$ may have changed by as much as 4 mM during changes in $[Cl^-]_o$. If $[K^+]_i$ is indeed as low as suggested above, the Donnan equilibrium theory (and using the above equation) predicts that this change is sufficient to account for the observed changes in $V_m$.

### *Potassium channels*

#### *Single channel $K^+$ currents in* Lymnaea *heart ventricle cells*

The resting conductance of excitable cell membranes is apparently universally mediated by ion channels which conduct primarily $K^+$ ions, but may also conduct other ion species to varying degrees[66]. Accordingly, the dependence of $V_m$ on potassium ions in *Lymnaea* implies that potassium-selective channels play an important role in the maintenance of the resting $V_m$. Single-channel (patch-clamp) examination of isolated *Lynmaea* heart ventricle muscle cells has revealed a potassium-conducting channel which, judging by its properties, is largely responsible for the resting conductance in these cells.

*Lymnaea* heart muscle cells were harvested by enzymatically disrupting heart ventricle fragments with trypsin and collagenase[17]. The mean resting $V_m$ of these cells was − 59 mV, which was very similar to the resting $V_m$ in the intact heart ventricle. This suggested that the isolated cells had not sustained significant damage during the isolation procedure. The isolated cells were initially rod-shaped with diameters of 5–10 μm and lengths of 40–120 μm. After about 7 days the cells de-differentiated to a spindle-shaped appearance (fig. 2). The results presented here were obtained from cells before they de-differentiated (1–4 days old). The effects of changes in the external $K^+$, $Cl^-$ and $Na^+$ concentrations on channel currents and conductances were examined using standard techniques[41]. Unless otherwise noted, the data presented below were obtained using the 'cell-attached' patch configuration.

Channel currents were evident only upon depolarization with normal *Lymnaea* saline (1.6 mM $K^+$) in the patch pipette. However, inward currents were readily seen at the resting $V_m$ with elevated intra-pipette $K^+$ concentration ($[K^+]_p$) (fig. 3). The predominant channel species, which was seen in virtually every patch, was characterized by a conductance of 33 pS and an $E_K$ of − 60 mV with normal $[K^+]$ in the patch pipette. A change in $[K^+]_p$ from 1.6 to 30 mM resulted in a 35 mV shift of $E_K$ in the depolarizing direction. The $E_K$ was about + 4 mV with 100 mM $[K^+]_p$. These results described a 27 mV slope per decade change in $[K^+]_p$ between 1.6 and 30 mM, and a slope of 57 mV per decade change between 30 and

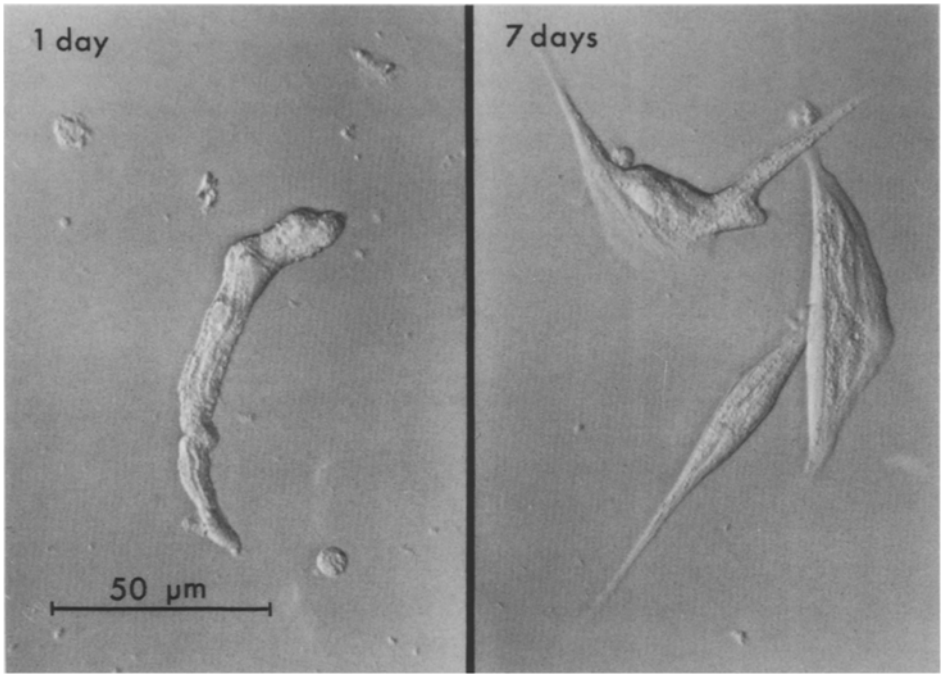


Figure 2. The appearance of enzymatically harvested *Lymnaea* heart cells at 1 day and 7 days following isolation. Note the spindle-shaped appearance of the older cells.

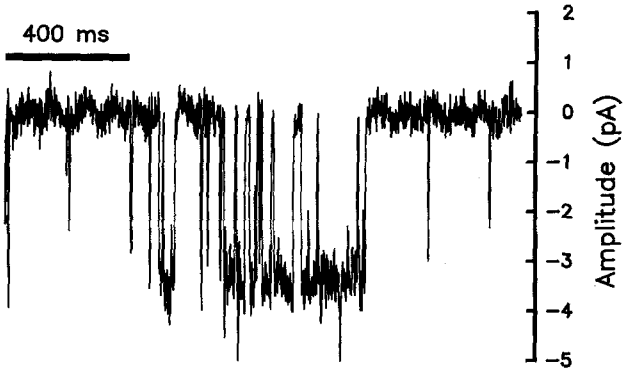


Figure 3. Spontaneous inward $K^+$ currents (downward deflections). The patch pipette $K^+$ concentration was 100 mM. The patch pipette potential was 0 mV ($V_m = -60$ mV).

100 mM. A change in $[K^+]_p$ from 1.6 to 100 mM was associated with a change in $[Cl^-]_p$ from 62.6 to 111 mM. If the channel was $Cl^-$-selective, a shift of $E_K$ in a hyperpolarizing direction would have been expected. Also, when the $[Cl^-]_p$ was kept constant during a change in $[K^+]_p$ from 1.6 to 30 mM, no shift in $E_K$ would have been expected for a $Cl^-$-selective channel. These results support the contention that this channel is primarily $K^+$-selective.

When bis(2-hydroxymethyl) dimethylammonium chloride was substituted for $Na^+$, the channel conductance increased from 33 to 37 pS and $E_K$ shifted from $-60$ to $-68$ mV. Furthermore, when $Na^+$ was absent in the patch pipette, outward currents were readily observed at the resting $V_m$, whereas outward currents were never seen with normal saline (50 mM $Na^+$). The hyperpolarizing shift in the reversal potential in the absence of $Na^+_p$ suggests that this channel is permeable to $Na^+$ as well as to $K^+$. Using the modified Goldman–Hodgkin–Katz equation (above), the measured reversal potential of $-60$ mV with normal (1.6 mM) $[K^+]_p$, and an estimated $[K^+]_i$ of 51.5 mM, gives a $P_K:P_{Na}$ ratio of about 16, which is close to the ratio of 14 estimated for *Lymnaea* heart ventricle cells in situ[15]. Given that this $K^+$ channel is open at resting $V_m$ (as seen with high $[K^+]$ saline in the patch pipette) and that $E_K$, the slope conductance and the $P_{Na}:P_K$ ratio for this channel are very similar to the in situ response to variations in $[K^+]_o$, it is likely that this channel is largely responsible for the resting conductance in *Lymnaea* heart ventricle cells.

Depending on the channel type, vertebrate $K^+$ channels are subject to block by tetraethylammonium (TEA), 4-aminopyridine (4-AP), quinidine, or a variety of monovalent or divalent cations applied to the inside or outside of the cell membrane[46]. Pharmacologically, the $K^+$ channel of *Lymnaea* does not fall neatly into any of the vertebrate $K^+$ channel categories. In contrast to mammalian $K^+$ channels, the *Lymnaea* $K^+$ channel was insensitive to both 4-AP and TEA applied to the outside of the membrane at concentrations as high as 10 mM. However, 0.25 mM quinidine gradually reduced the $K^+$ channel amplitude in an apparent 'use-dependent' manner. The quinidine block was complete after several seconds of application[17]. Also, 50 mM $Ba^{2+}$ blocked the channel (unpublished observations). Furthermore, the *Lymnaea* $K^+$ channel does not appear to be $Ca^{2+}$-activated.

Apamin, which blocks $Ca^{2+}$-activated $K^+$ channels in vertebrate muscle cells[55] and cetiedil, which blocks a $Ca^{2+}$-sensitive $K^+$ conductance in blood lymphocytes[78] had no effect on the *Lymnaea* $K^+$ channel. In addition, $K^+$ currents could be resolved in 'inside-out' patches exposed to EGTA/$Ca^{2+}$ saline with a $Ca^{2+}$ concentration of $10^{-8}$ M[82]. Given this pharmacological profile, the *Lymnaea* $K^+$-channel most closely resembles the vertebrate delayed rectifier $K^+$ channel (cf. Hille[46]).

Dorsett and Evans[29] examined $K^+$ and $Ca^{2+}$ currents in muscle cells of the mollusc *Philine aperta* using the two-electrode voltage clamp technique. They resolved three outward potassium currents and an inward current which was abolished by zero-calcium salines and the $Ca^{2+}$ entry blockers $Cd^{2+}$ and verapamil. This suggests that in *Philine*, there are at least three types of $K^+$ channel and at least one $Ca^{2+}$ channel type. Although all of the channels in *Lymnaea* heart ventricle have not been characterized, other channel currents which might be mediated by $K^+$, $Cl^-$, and possibly $Na^+$ have been observed[33, 74, 82]. The resolution of these channel types must await further investigation. The presence of these various currents, however, underscores the complexity of the membrane permeability in *Lymnaea* heart muscle, and it may very well be that more than one type of $K^+$ current is present in this muscle.

*$K^+$-channel kinetics and stretch-sensitivity*

Our contemporary view envisages channels as enzyme-like protein molecules which catalyze the transfer of ions from one side of the cell membrane to the other. A basic tenet is that ion channels are not simply 'holes' in the membrane which allow the free passage of ions, but rather that channels can discriminate between different ion species. As such, ion channels must contain inherent energy barriers which are more or less favourable for a particular ion species. The passage of an ion through a channel would, therefore, be subject to well-established laws of kinetic theory. Consequently, ion channels must have states which would be defined by ion-channel interactions. These kinetic states can be quantified by an appropriate analysis of single channel current records[41].

Open- and closed-time distributions were analyzed to determine the number of kinetically distinguishable states which the *Lymnaea* $K^+$ channel could assume. The distribution of open times was best fitted by two exponentials with time constants of 0.21 and 2.50 ms. The closed-time distribution was best fitted by three exponentials with time constants of 0.13, 0.69 and 8.33 ms[82]. This albeit rather complex kinetic scheme would not appear to be unusual for an ion channel except for the fact that the activity of the channel was dependent on the degree of membrane stretch. That is, the probability of this $K^+$ channel being open ($P_{(open)}$) increased dramatically with the application of suction to the patch pipette (fig. 4). An analysis of the relationship between membrane stretch, single channel conductance and the open and closed time

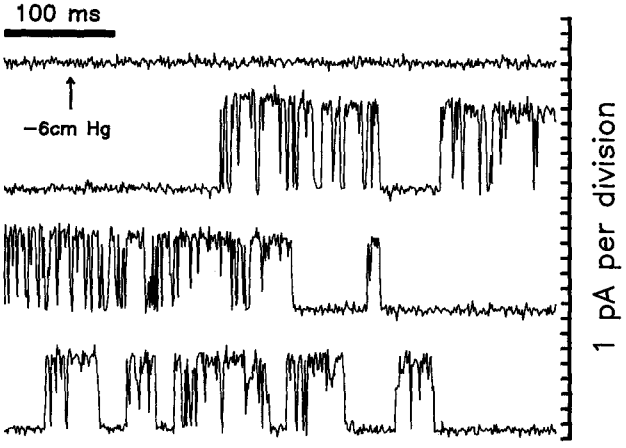


Figure 4. The effect of membrane stretch on *Lymnaea* $K^+$ channel activity. The arrow marks the beginning of the application of − 6 cm Hg suction to the patch pipette. The patch pipette contained 59 mM $Na^+$ and 1 mM EGTA. The pipette potential was − 80 mV ($V_m$ = + 20 mV). Upward deflections indicates outward currents.

constants was undertaken in order to resolve the mechanosensitive kinetic component of this channel. Since current/voltage curves generated in the presence and absence of suction were indistinguishable, it was evident that membrane stretch did not affect the rate-limiting component for ion permeation. It was, therefore, assumed that the increase in $P_{(open)}$ was due to a change in one or more of the kinetic states of the channel.

The comparison of the various kinetic states of the channel in the absence and presence of suction revealed that only the longest-lived closed state was affected by membrane stretch[82]. That only one of the several channel types observed responded to stretch with an increased activity under normal ionic conditions, and that only one of the kinetic states of the $K^+$ channel was affected, suggested that this phenomenon was not an artefact of the patch-clamp technique. Moreover, mechano-sensitive channels have been previously observed in a number of vertebrate preparations[9, 26, 36]. The most fully characterized of these occurs in embryonic chick skeletal muscle[36, 37], although the vertebrate mechano-sensitive channels appear to discriminate rather poorly between $K^+$ and $Na^+$, in contrast to the *Lymnaea* $K^+$ channel. However, it must be noted that no physiological function has yet been ascribed to the mechano-sensitivity of certain ion channels. Indeed, it would appear that, at least in *Lymnaea* neurons, $K^+$-channel mechano-sensitivity might well be an artefact[73]. For a comprehensive overview of mechano-sensitive channels and their possible physiological significance, the reader is referred to the recent review by Morris[72].

There is evidence that protein kinase C might also be involved in the modulation of *Lymnaea* K channels. The application of $10^{-6}$ M phorbol myristate acetate, a potent stimulator of protein kinase C[56], to the cells outside of the patch pipette in the absence of pipette suction promoted $K^+$ channel single currents. That these cur-

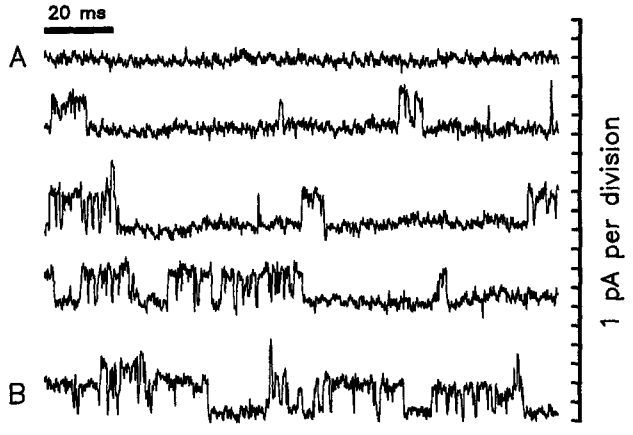


Figure 5. Activation of potassium currents in *Lymnaea* heart cells by $10^{-6}$ M phorbol myristate acetate. *A* Contiguous traces of channel activity in the absence of suction. PMA was applied to the cell about 2 s before the beginning of the first trace. *B* Increased channel activity following the application of − 4 cm Hg suction to the patch pipette. Upward deflections represent outward currents. The patch pipette potential was 0 mV ($V_m$ = − 60 mV).

rents were passing through the stretch-sensitive $K^+$ channels was evidenced by the additional increase in activity when suction was applied to the patch pipette (Brezden and Gardner, unpublished observations; fig. 5).

*Calcium channels*

*Pharmacological evidence for the existence of $Ca^{2+}$ channels in* Lymnaea *muscle*

An agent which has proven to be a very useful probe for examining the membrane properties of both *Lymnaea* neurons and muscles is the molluscicide Frescon (N-triphenylmethyl morpholine). The action of Frescon in *Lymnaea* neurons and muscle cells has been comprehensively reviewed[32], but some of the more interesting aspects of this molluscicide with regard to $Ca^{2+}$ permeability in *Lymnaea* muscle will be summarized here.

An apparatus was used which allowed the measurement of tension in perfused whole heart, or the penis retractor muscle (PRM). The application of $10^{-5}$ to $10^{-6}$ M Frescon induced an irreversible contracture of the *Lymnaea* heart ventricle and PRM within 30 s. In whole heart preparations this contracture reached a maximum and subsequently declined to a residual tension which was above the pre-treatment level. In the PRM and heart ventricle strips the contracture was maintained at close to maximal levels. The difference in response by the intact ventricle appeared to be a function of the elastic properties and arrangements of muscle fibres[11].

It was found that the Frescon-induced contracture could be prevented by a low $Ca^{2+}$/high $Mg^{2+}$ saline, $Ni^{2+}$, $Co^{2+}$, $Mn^{2+}$ or $La^{3+}$. The organic $Ca^{2+}$ blocker D-600 was also effective in blocking Frescon action, albeit at the relatively high concentration of $10^{-4}$ M. Furthermore, a Frescon-induced contracture was not affected by the depletion of intracellular $Ca^{2+}$ stores after repeated applications of caffeine[12,13]. These results suggested that external $Ca^{2+}$ is required for Frescon action, and that this molluscicide promotes an irreversible influx of $Ca^{2+}$. Since Frescon does not act as an ionophore[14], it is reasonable to assume that the action of this molluscicide can be attributed to the activation of one or more types of $Ca^{2+}$ channel.

There is electrophysiological and pharmacological evidence for the existence of distinct potential-dependent $Ca^{2+}$ (PDC) and receptor-operated $Ca^{2+}$ (ROC) channels in vertebrate cells[8,93]. Until recently, the evidence for the existence of ROC channels was rather indirect and it has been argued that the distinction between ROC and PDC channels is artificial as both receptor operation and potential dependence can exist in the same channel[69]. At least some ROC channels have been shown to be activated independently of changes in $V_m$ by chemical agents or transmitters which may act directly on the channel[4], or indirectly via activation of secondary messenger systems[30,64,67,95]. PDC channel permeability, on the other hand, responds to changes in $V_m$, although PDC channel activity can also be modulated by chemical agents[87]. Certain $Ca^{2+}$ channel blockers seem to be more specific against one or the other type of $Ca^{2+}$ channel. For instance, PDC channels can be blocked by sodium pentobarbital (NaPB)[7] and can be activated by the depolarizing action of high $[K^+]_o$[8,12]. ROC channels can be blocked by sodium nitroprusside (NaNP)[93]. In *Lymnaea* PRM, $5 \times 10^{-3}$ M NaPB blocked high-$K^+$-induced contracture but did not prevent Frescon-induced contracture. On the other hand, $10^{-2}$ M NaNP completely abolished the Frescon-induced contracture in heart ventricle strips and reduced the contracture in the PRM by about 50%. However, NaNP had no effect on high-$K^+$-induced contracture in either the PRM or heart ventricle. Also, Frescon-induced contractures, and contractures induced by high $[K^+]$ saline, were superimposable[16]. Furthermore, Frescon-induced contracture in *Lymnaea* heart muscle was not accompanied by a depolarization of $V_m$[11]. These results suggested that both PDC and ROC channels might exist in *Lymnaea* heart muscle and that Frescon might act on ROC channels rather than high-$K^+$-activated PDC channels.

*Divalent cation channels in* Lymnaea *heart ventricle cells*

Single-channel inward current could not be detected in isolated *Lymnaea* heart ventricle cells when the patch pipette contained normal saline, even with depolarizing voltage steps. This was quite unexpected as the pharmacological evidence for the existence of $Ca^{2+}$ channels was rather compelling. However, removal of divalent ions from the patch pipette had been shown to promote monovalent ion conductance through $Ca^{2+}$ channels in vertebrate cells[42,88]. It was considered that such might be the case for isolated *Lymnaea* heart cells as well. Indeed, when only 59 mM $Na^+$ and 1 mM EGTA were present in the patch pipette (giving an estimated patch pipette $Ca^{2+}$ concentration of only 3.2 nM) several

types of inward currents were observed, including a voltage-gated single-channel current and two spontaneously active 'non-voltage-gated' channel types which conducted ion currents over a broad range of $V_m$. The spontaneously active channels, which could be distinguished by different conductances, were labelled SG ('small conductance') and LG ('large conductance') channels. In addition, single channel $Ba^{2+}$ and $Ca^{2+}$ currents could be detected when only 50 mM $Ba^{2+}$ or 50 mM $Ca^{2+}$ was present in the patch pipette. However, although single channel $Ca^{2+}$ currents were seen with only 50 mM Ca in the patch pipette, it was very difficult to obtain good pipette-membrane seals under these conditions. Consistently good seals could much more easily be obtained with $Ba^{2+}$ in the patch pipette[33]. Thus, only the two non-voltage-gated channels and the $Ba^{2+}$-permeable (BaP) channel have been well-characterized so far[18]. Further discussion will be restricted to these three channel types.

The single-channel inward current, whether carried by $Na^+$ or $Ba^{2+}$, usually disappeared within a few seconds of seal formation, or tapered off at various rates, seldom lasting longer than a few minutes. This hindered the kinetic analysis of these channels, but eventually enough records were obtained to allow characterization of these channel types.

*The large-conductance (LG) channel*

The LG channel (fig. 5A) was the most readily detected channel type, occasionally remaining spontaneously active for several minutes. This channel did not appear to be inactivated by either depolarization or hyperpolarization of the $V_m$ as channel currents were seen at membrane potentials between $-110$ and $-40$ mV. When activity ceased, the channels could not be re-activated by hyperpolarization/depolarization voltage steps. Thus the channel did not appear to be 'voltage-gated' since it was not activated by changes in $V_m$. However, over this broad range of potential change, our $P_{(open)}$ data did indicate a weak 'voltage-sensitivity' in that the channel activity increased with depolarization[18].

The LG channel exhibited at least two conductance states as evidenced by single-channel records showing direct transitions from one amplitude to the other (fig. 6). The amplitude of the LG channel currents at resting $V_m$ was about $-2$ pA for the smaller conductance state and $-3$ pA for the larger. The slope conductance was 50 pS for the smaller conductance state and 72 pS for the larger. The extrapolated reversal potentials for both subconductances were nearly identical at about $-20$ mV (fig. 7). The open dwell-time distributions for either conductance state were best fitted by the sum of two exponential terms, which were essentially identical in both conductance states, averaging 0.5 and 1.8 ms. Kinetic analysis of the closed dwell-time data revealed three closed-time constants with values of 0.2, 1.4 and 10.5 ms in both conductance states[18].

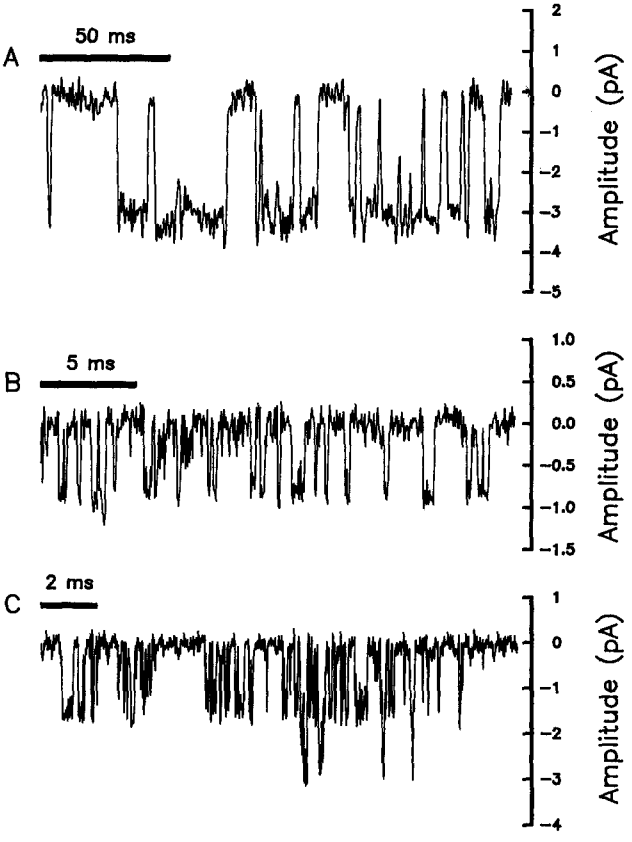


Figure 6. *A* Spontaneously active LG channel currents. Patch pipette contained 59 mM $Na^+$ and 1 mM EGTA. *B* Spontaneously active SG channel currents. Patch pipette contained 59 mM $Na^+$ and 1 mM EGTA. *C* Spontaneously active BaP channel currents. Patch pipette contained 50 mM $Ba^{2+}$. The patch pipette potential was 0 mV ($V_m = -60$ mV) for all three records. Note differences in the scales. Downward deflections represent inward currents.

*The small-conductance (SG) channel*

The SG channel was seen in about 10% of the patches (fig. 5B). It was often characterized by infrequent bursts of activity separated by long periods of inactivity. At resting $V_m$ the amplitude of the SG channel was about $-1$ pA. The slope conductance was about 15 pS and the extrapolated reversal potential was about $-10$ mV (fig. 7). Like the LG channel, the SG channel also did not appear to be inactivated by changes in $V_m$ but it did show a voltage dependence in that SG channel activity tended to increase with membrane depolarization. The open dwell-time distribution was best fitted by a single exponential, suggesting one open state for this channel. Closed dwell-times measured within bursts were best fitted by two exponential terms with time constants of about 0.3 ms and 1.8 ms. There probably is a third closed time constant which defines the closed dwell-times between bursts but, as the SG bursts were infrequent, there were too few events to measure this accurately[18]. Since the kinetics of the SG channel are quite different from the LG channel kinetics it is not likely that the SG channel represents yet another subconductance state of the LG channel, but rather that it is a separate channel type.

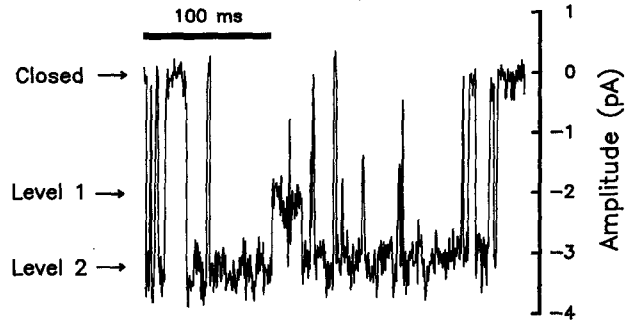


Figure 7. Spontaneous transition of LG channel currents between two amplitudes. The patch pipette potential was 0 mV ($V_m = -60$ mV). Downward deflections represent inward currents.

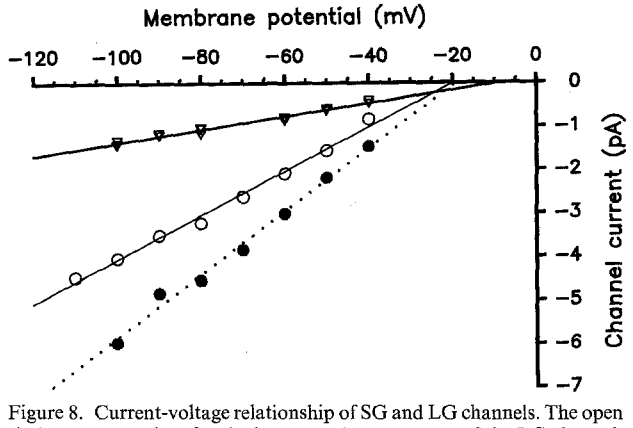


Figure 8. Current-voltage relationship of SG and LG channels. The open circles represent data for the lower conductance state of the LG channel. The closed circles are data from the higher LG channel conductance state. The open triangles represent data from the SG channel. The patch pipette contained 59 mM $Na^+$ and 1 mM EGTA. The LG channel data are from a single patch. The SG channel data are combined from 5 patches.

*$Ba^{2+}$ conductance*

With 50 mM $Ba^{2+}$ in the patch pipette, the mean unitary current amplitude was about − 1.3 pA at the resting $V_m$ (fig. 5C). The BaP channels, which also did not appear to be voltage-gated, were detected at membrane potentials between − 80 and − 20 mV. The slope conductance was about 12 pS and the extrapolated current/voltage curve defined a reversal potential of about + 55 mV (fig. 8). The open dwell-times were best fitted by a single exponential term with a time constant of about 1.4 ms. As in the case of the SG channel, the $Ba^{2+}$ currents also appeared in infrequent bursts. Closed dwell-times, measured only within those bursts where no superimposed opening occurred, were best fitted by two exponential terms with time constants of 0.3 ms and 2.2 ms. The between-burst closed times were not analyzed because of the small number of these events[18].

*Are LG, SG and BaP channels $Ca^{2+}$ channels?*

In the absence of patch pipette $Ca^{2+}$, the inward LG and SG currents were not passing through $K^+$ channels as they were observed simultaneously with outward $K^+$ channels[33]. The currents detected with only 50 mM $Ba^{2+}$ in the patch pipette could not be due to the influx of a different cation species since only $Ba^{2+}$ was present to carry the inward current. Neither could SG, LG or BaP currents be carried by an outward $Cl^-$ current because the reversal potentials under these conditions were much more positive than the $Cl^-$ equilibrium potential, which is close to the resting $V_m$.

The reversal potentials for the SG and LG $Na^+$ currents were considerably more negative than the reversal potential for the BaP channel currents. Furthermore, the SG and LG channel currents reversed at a potential which was much more negative than would be expected for a $Na^+$ channel. It appeared that the reversal potential for both SG and LG channel currents rested somewhere between $E_{Na}$ and $E_K$. This could be explained if the $Na^+$ current carried by the SG and LG channels was passing through a symmetrical $Ca^{2+}$ channel. In this case, a counter-flux of $K^+$ ions could effectively shift the reversal potential for the $Na^+$ currents in a negative direction towards $E_K$[43].

The evidence which supports the contention that SG and LG channels may be $Ca^{2+}$ channels is summarized as follows: 1) the channels conduct $Na^+$ only in the absence of $Ca^+$ which would be expected if the $Ca^{2+}$ channel binding sites had a much lower affinity for $Na^+$ than for $Ca^{2+}$; 2) SG and LG channels are blocked by very low concentrations of $Ca^{2+}$[33]; 3) the channel $Na^+$ currents are blocked by $Cd^{2+}$ and $Co^{2+}$, which are known $Ca^{2+}$ channel blockers[33]; 4) $Ca^{2+}$ and $Ba^{2+}$ currents with highly positive reversal potentials *do* exist in *Lymnaea* heart ventricle cells; and 5) the reversal potentials for SG and LG currents were much more negative than would be expected for a $Na^+$-selective channel. This cumulative evidence suggests that the SG and LG $Na^+$ currents passed through a symmetrical $Ca^{2+}$ channel which also allowed an outward counterflux of $K^+$[18].

The open and closed dwell-times of the BaP channel most closely resembled those of the SG channel. However, the expectation was that $Na^+$ permeability would have been greater than that of divalent cations in the absence of patch pipette $Ca^{2+}$ if both $Ba^{2+}$ and $Na^+$ were passing through the same channel (cf. Hess et al.[43]). This was not the case. On the other hand, the LG channel, which exhibited a greater $Na^+$ permeability than the SG channel, had different kinetic properties than the BaP channel. On the basis of the available evidence, therefore, it is not possible to conclude that the BaP channel and either the SG or the LG channel are one and the same.

In view of the hypothesized mode of action for Frescon (see above), it would be expected that this agent might activate SG, LG or BaP channels. However, the application of Frescon to the cell outside the patch failed to elicit any response (Brezden and Gardner, unpublished observations). Since Frescon is very hydrophobic and thus forms particulate suspensions (with particle sizes of up to 10 μm[10]) it could not be included in the patch pipette. Consequently, we could not investigate a possible direct activation of the putative $Ca^{2+}$ channels by Frescon us-

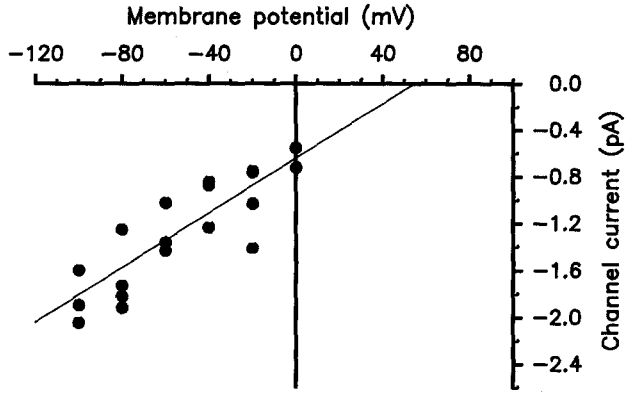


Figure 9. Current-voltage relationship of the BaP channel. The patch pipette contained 50 mM $Ba^{2+}$. Combined data from 4 patches.

ing the patch clamp technique. The question of a possible direct action of Frescon on these channels is, therefore, still unresolved, but under continued investigation.

*The peptide FMRFamide activates SG, LG and BaP channels*

The tetrapeptide Phe–Met–Arg–Phe–$NH_2$ (FMRFamide) and related peptides have recently been localized in a pair of motoneurons in *Lymnaea*. Stimulation of these neurons has been shown to accelerate the heart beat rate, and it was suggested that this cardioexcitatory action is mediated by FMRFamide[6,21]. Following the discovery of putative $Ca^{2+}$ channels in the sarcolemma of *Lymnaea* heart muscle[18], it was reasonable to question whether or not changes in the activity of the heart caused by the stimulation of *Lymnaea* motoneurons might not be mediated by the modulation of one or more of these channels. This question could be indirectly approached by examining single channel activity in isolated heart cells during superfusion with FMRFamide.

Superfusion of the heart cells with $10^{-6}$ M FMRFamide over areas away from the patch pipette induced a large increase in channel activity after a delay of about 5 s (fig. 9). The reversal potentials, slope conductances and open dwell-times of the FMRFamide-activated channels were essentially identical to those of the spontaneously active SG and LG channels. The $P_{(open)}$, calculated over 30-s intervals before and after the onset of peptide-induced activity, increased by a factor of about 1500. Simultaneous activation of several channels in the patch was reflected by the increased frequency of multiple openings. The increased activity persisted for 1–4 min, then gradually declined until channels became relatively quiescent with only occasional openings over the course of about 1 h, which was as long as reliable seals could be maintained[19].

With 50 mM $Ba^{2+}$ in the patch pipette, extra-patch superfusion with $10^{-8}$ M FMRFamide resulted in the appearance of inward $Ba^{2+}$ currents (fig. 10). The $P_{(open)}$ (calculated as above) increased by a factor of about 500.

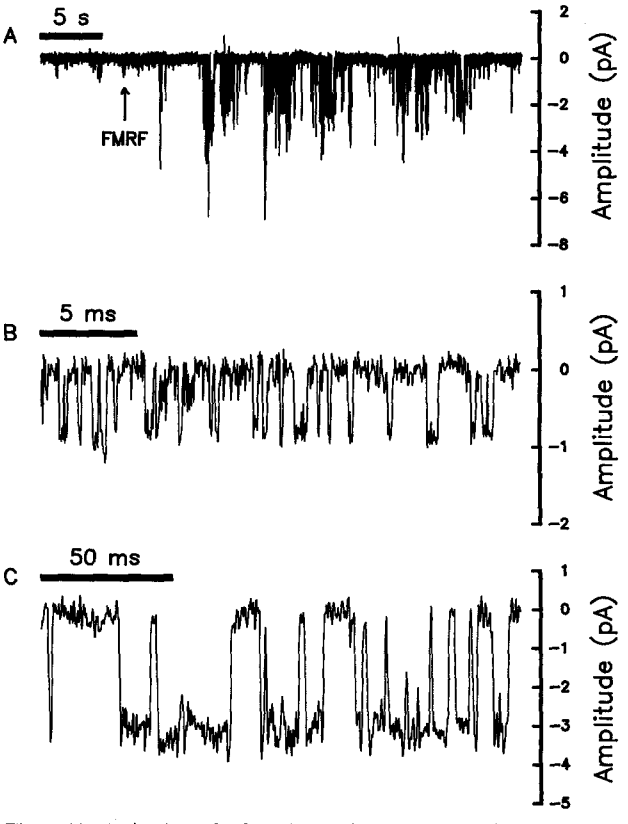


Figure 10. Activation of SG and LG channel currents by extra-patch superfusion with $10^{-6}$ M FMRFamide. The patch pipette contained 59 mM $Na^+$ and 1 mM EGTA. The patch pipette potential was 0 mV ($V_m = -60$ mV). Downward deflections represent inward currents. *A* The SG events are those with an amplitude of about $-1$ pA. The LG events have an amplitude of $-2$ to $-3$ pA. Deflections having higher amplitudes represent multiple openings. The occasional upward excursions from the baseline (outward currents) are $K^+$ currents which occasionally open spontaneously in the absence of patch pipette $K^+$. *B* FMRFamide-activated SG channel events on a shorter time scale. *C* FMRFamide-activated LG channel events on a shorter time scale.

The FMRFamide-activated $Ba^{2+}$ currents had a reversal potential, slope conductance and open dwell-time constants which were very similar to the spontaneously active BaP channel currents[19].

The activation of SG, LG and BaP channels by FMRFamide applied outside the patch, and the several second latency before the response, suggests that the action of this peptide is mediated by a second messenger. It is very unlikely that the water-soluble FMRFamide could have diffused through the electrically tight seal between the cell membrane and the pipette glass to activate the receptors directly. In *Aplysia* sensory neurons, FMRFamide-induced activation of S-type $K^+$ channels[20,91] may be mediated by arachidonic acid metabolites[91]. Whether the same messenger might activate the $Ca^{2+}$ channel in *Lymnaea* is currently under investigation. At present it does not seem likely that protein kinase C is involved in the FMRFamide response as phorbol myristate acetate (with only 59 mM $Na^{2+}$ and 1 mM EGTA in the patch pipette) did not induce either SG or LG channel activity,

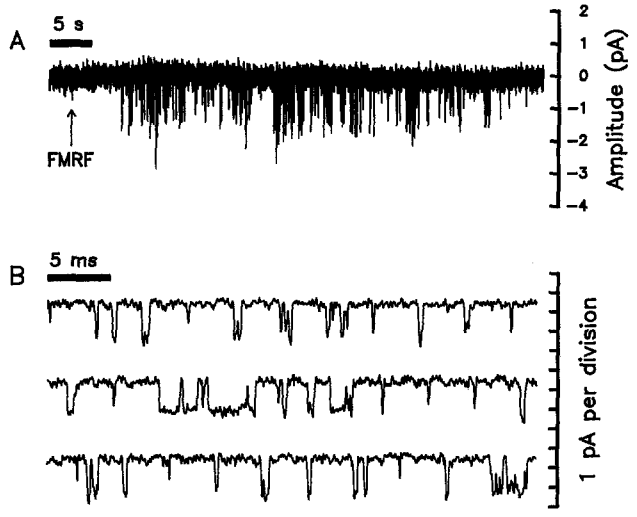


Figure 11. *A* Activation of unitary $Ba^{2+}$ channel currents by extra-patch superfusion with $10^{-8}$ M FMRFamide. The patch pipette contained 50 mM $Ba^{2+}$. The patch pipette potential was 0 mV ($V_m = -60$ mV). *B* FMRFamide-activated $Ba^{2+}$ currents shown on a shorter time scale. Downward deflections represent inward currents.

but did promote an increase in the activity of the $K^+$ channel (fig. 5).

It would appear that the neuronal control of the *Lymnaea* heart is comparable in complexity to the mammalian heart with regulation dependent on a variety of peptidergic and non-peptidergic neurons[21–23,35]. Although the coordinated action of this regulation is not well-understood, the above evidence suggests that the myogenic heart of *Lymnaea* contains an extensive array of different channel types which may be modulated by one or more types of second messenger. The characterization of these channel types and the resolution of their mode of modulation is at an early stage. However, it is hoped that a better understanding of the types of channels in *Lymnaea* heart muscle will provide a foundation for examining possible synergistic actions of putative modulatory agents and their mode of action at the cell membrane level.


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0014-4754/92/090841-12$1.50 + 0.20/0


# Are stretch-sensitive channels in molluscan cells and elsewhere physiological mechanotransducers?

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*Abstract.* Single-channel recordings of dozens of cell types, including invertebrate (molluscan) and vertebrate heart cells, reveal stretch-sensitive ion channels. The physiological roles of these channels are undoubtedly diverse but it is usually assumed that the roles they play are related to the channels' mechanosensitive gating. Whether this assumption is valid remains to be seen. Attempts to connect the single-channel observations with the mechanical aspects of physiological or developmental processes are discussed. In the case of molluscan cells, recent work suggests that their stretch channels have physiological functions unrelated to mechanosensitive gating.
*Key words.* Stretch; mechanosensitive; ion channel; physiological role.

## *Introduction*

Ion channels sensitive to tension may be important for cytomechanics and tissue mechanics, topics germane to the control of circulation in invertebrates. I will discuss stretch-activated (SA) ion channels, but the focus will be broader than their possible involvement in circulatory control; my purpose is to ask, "How good is the evidence implicating SA ion channels in mechanotransduction in any physiological process?"

About two years after the initial single-channel study of stretch-activated (SA) channels, which were nonselective cation (SACat) channels of vertebrate skeletal muscle[20,46], SA $K^+$ (SAK) channels were described. They were found in molluscan heart[8,50], and being $K^+$-selective rather than nonselective, they constituted a second general type of SA channel. Given that molluscan heart is both mechanically active and stretch-sensitive[26], it was tempting, having found SAK channels uniformly distributed in the ventricular cells at $\sim 1\ \mu m^{-2}$, to speculate about special cardiac functions which they might serve. Subsequently, however, we showed that SAK channels in molluscs are not restricted to heart cells; essentially identical channels are ubiquitous in the neurons of gastropods[3,40]. Insect somatic muscle, too, has comparable SAK channels[19,61], and SAK channels have been described in diverse vertebrate cell types[41].

In fact, SA channels of various selectivities have now turned up in most cell types (bacteria, yeast, plant, animal)[41,47] and suggestions about their possible functions have been legion. Membrane tension induced by suction during single-channel recording does not affect most channel types (see, for example, Zagotta et al.[61]). The effect of tension on the 'open probability' of SA channels, however, is so striking that it is natural to presume that the channels act as mechanosensor/effectors. A continual mechanical threat to all cells, colloidal osmotic stress, is exacerbated by osmotic perturbations. Hence, an evolutionarily ancient SA channel-based mechanism for swelling-activated electrolyte loss (using the channels both as sensor and effector in the simplest case) would make sense a priori[31,48] and help account for the phenomenon of hyposmotic-shock-induced increased permeability to ions[9].

This is an appealing notion, but to date, no experimental evidence conclusively links stretch channel activity to osmoregulation or to any other cell physiological process. At the outset we need to deal with the reductionist's