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Characterization of the voltage-activated currents in cultured atrial myocytes isolated from the heart of the common oyster *Crassostrea gigas*

Jean-Pierre Pennec*, Hélène Talarmin, Mikaël Droguet, Marie-Agnès Giroux-Metgès, Maxime Gioux and Germaine Dorange

EA 3879, Unité de Physiologie Comparée et Intégrative, Institut de Synergie des Sciences et de la Santé, 22 avenue Camille Desmoulins, CS93837, 29238, Brest-cedex 3, France

*Author for correspondence (e-mail: jpennec@univ-brest.fr)

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Summary

Using the macro-patch clamp technique, we show that cardiac myocytes isolated from the heart of the oyster *Crassostrea gigas* possess several types of voltage-activated ionic currents. (1) A classical non-inactivating potassium current of the I_K type that is inhibited by tetraethyl ammonium and shows an outward rectification and a slow activation. (2) A potassium current of the I_A type that shows rapid activation and inactivation, and is blocked by 4-amino pyridine or preliminary depolarisation. (3) A potassium calcium-dependent current that is inhibited by charybdotoxin, activated by strong depolarisations and shows a large conductance. (4) A calcium inward current of the L-type that is inhibited by verapamil, cobalt and high concentrations of cadmium. This current is identified in most cells, but a T-type calcium current and classical fast sodium current are only identified in few cells, and only after a strong hyperpolarizing pulse. This suggests that these channels are normally inactivated in cultured cells and are not involved in the spontaneous activity of these cells. When they exist, the fast sodium channel is blocked by tetrodotoxin. The L-type calcium conductance is increased by serotonin. The identification in cultured oyster atrial cells of classical ionic currents, which are observed in most vertebrate species but only in a few species of molluscs, demonstrates that these cells are an interesting model. Moreover the viability and the electrophysiological properties of these cells are not significantly modified by freezing and thawing, thus increasing their usefulness in various bioassays.

Key words: bivalve, oyster, *Crassostrea gigas*, heart, ionic current, cell culture, patch clamp, bioassay, cryopreservation.

Introduction

There have been few studies on the electrophysiological properties of bivalve heart cells and particularly on the properties of ionic channels underlying spontaneous activity. There is only one report of the existence of voltage-dependent sodium-, calcium- and potassium channels in cardiac myocytes isolated from the blue mussel heart (Curtis et al., 1999). The ionic basis of cardiac activity in the bivalves *Modiolus demissus* (Wilkens, 1972) and *Perna perna* (Peirera Ferreira and Salomão, 2000) has been described but without a characterization of the ionic channels. Brezden and Gardner (1992) showed the existence of ionic channels in the heart cells of the snail *Lymnaea stagnalis*. More recently, two types of potassium channels (Yeoman and Benjamin, 1999) and also two types of calcium channels (Yeoman et al., 1999) were characterized in the heart of the same species. Three major potassium currents were identified in a preliminary report in the squid *Loligo forbesii* (Ödblom and Williamson, 1995), followed more recently by another species, *Allotheutis subulata* (Ödblom et al., 2000): a fast inactivating current that is inhibited by 4-amino pyridine (4-AP), a delayed outward rectified current that is inhibited by tetraethyl ammonium chloride (TEA), and a calcium-activated current that is inhibited by cobalt ions and apamin. Calcium and fast sodium channels were also found. To our knowledge, ionic channels in cells isolated from the heart of the common oyster *Crassostrea gigas* have not been described before.

Some studies have shown that it is possible to isolate and cultivate cells from bivalves, especially from bivalve heart (Curtis et al., 1999; Le Marrec et al., 1999; Pennec et al., 2002). There are few reports concerning fundamental electrophysiological studies in such cells, however, and the characterization of the ionic currents in the heart cells of the oyster remains to be done. In a previous study (Pennec et al., 2002) we showed that it is possible to isolate viable cardiomyocytes from the heart of the oyster and to culture them for longer periods (several days to a few weeks), and to perform electrophysiological studies in these cells using a macro-patch clamp technique. These cells were spontaneously beating after a few days of culture and formed

contractile networks in the culture dishes. The beating rhythm could be decreased by acetylcholine and increased by epinephrine. These effects could be blocked by atropine and propranolol; this shows that the cells have functional cholinergic and adrenergic receptors. In addition, we showed that a significant reduction of beating rate was induced by tributyltin (TBT) and cadmium (Cd). These effects were attributed to a reduction of the inward current that is thought to be mainly a calcium current. However, there was no precise characterization of the different ionic currents and, hence, the identification and determination of the characteristics of the main ionic currents in these cells remained to be carried out. Similarly, the role of the different channels in the determination of the spontaneous beating rate of the isolated cells was unknown. Besides such fundamental aspects, a better knowledge of these channels could be helpful for determining the level of toxicity of sublethal, low concentrations of marine pollutants. The aims of the present paper were (1) to characterize the main ion channels in cultured cells isolated from the atrium of the oyster heart and to compare these channels to those already described in other mollusc cells; (2) to investigate the role of these channels in the modulation of spontaneous contractions observed in the cultured cells; and (3) to demonstrate that freezing and thawing does not significantly change the electrophysiological properties of the cells, so enabling the use of thawed cells as a useful bioassay for various pharmacological or ecotoxicological studies.

Materials and methods

Experiments were performed on cultured cells isolated from the atrium of the heart of common oysters *Crassostrea gigas* Thunberg 1793. collected from a local fish farm. The oysters were stored in laboratory tanks filled with circulating aerated sterile seawater prior to experiments, and fed with micro-algae. The isolation technique of the cells was previously described (Pennec et al., 2002); briefly, the oysters were washed with sterile seawater and the outside of the shell was rapidly sterilized with 70% ethanol. They were opened under sterile conditions and the heart carefully dissected out. The atrium was minced and then placed in Hanks' balanced salt solution with 0.025% Pronase (w/v) for 12 h at 4°C. The dissociated cells were filtered through a 60 μm nylon mesh, washed twice and placed in a culture medium containing sterile seawater supplemented with 10% Leibovitz medium L15, foetal calf serum, 10^{-2} mol l^{-1} Hepes buffer, and antibiotics: penicillin–streptomycin (50 i.u. ml^{-1} and 50 μg ml^{-1}, respectively), kanamycin (10 μg ml^{-1}) and gentamycin (20 μg ml^{-1}). The pH was adjusted to 7.3 and the osmolarity was 1100 mOsm. The percentage of viable isolated cells, evaluated by a Trypan Blue exclusion test, was better than 95%. Cells (3.5×10^6) were seeded in each Petri dish (Falcon, Becton Dickinson, Le-Pont-de-Claix, France; diameter: 35 mm) then incubated at 18°C before the experiments. Most of the experiments were carried out after 1 week of culture.

Cryopreservation of the cells

Cells were frozen in 12% dimethylsulfoxide (DMSO) containing Leibovitz medium at a cooling rate of approximately 2–3°C min^{-1}, down to –80°C. After 2 h, the cells were placed in liquid nitrogen where they were stored until thawing. This technique has been extensively described by Le Marrec-Croq et al. (1998). After thawing, DMSO containing medium was removed and the cells were resuspended in normal culture medium, then seeded in 35 mm Petri dishes and cultured at 18°C as described above. The percentage of viable thawed cells was evaluated by a Trypan Blue exclusion test.

Electrophysiological technique

A macro-patch clamp technique (see Malecot and Duval, 1992) was used to record the membrane currents of the cultured cardiomyocytes under voltage-clamped conditions in the cell-attached configuration. Pipettes were pulled from borosilicate glass capillaries (GC150 TF10; Clark Electromedical, Phymep, Paris, France) using a microprocessor-controlled puller (DMZ, München, Germany). They were automatically heat polished. The diameter of the opening (3.3±0.02 μm) was checked by electron microscopy. The average resistance of pipettes filled with the standard medium (modified seawater) was 0.8 MΩ. Junction potential was corrected before realization of a seal. The tip of the pipette was positioned in contact with the cell membrane using a hydraulic micromanipulator (Narishige, Tokyo, Japan), and a moderate suction was applied to induce the formation of a seal better than 1 GΩ (gigaseal); thereafter the depression was released. Patches showing either bleb formation or unstable seal values were discarded. The formation of the seal and the capacitance compensation were monitored on an oscilloscope (TDS 340A; Tektronics, Beaverton, OR, USA). Recordings were made at room temperature (averaging 20°C). The resting membrane potential of the cells was previously measured using an intracellular microelectrode filled with 2.7 mol l^{-1} KCl (diameter <1 μm, average resistance 40 MΩ). The microelectrodes were connected to the amplifier (Axon; Geneclamp 500 B, Foster City, CA, USA) *via* a headstage designed for voltage measurement (HS-2A; Axon). Current measurements were made using a patch-clamp amplifier (Geneclamp 500 B) equipped with a current-to-voltage converter headstage (CV5 series; Axon). The outputs (voltage and current) were connected to the scope and to a micro-computer (PC compatible) *via* an analog-to-digital converter (CED 1401 plus; Cambridge, UK) running at 125 kHz. A program (WCP v. 3.06 from Strathclyde University, Scotland, UK) was used to record the currents and to deliver sequences of programmed voltage pulses *via* the analog output of the CED to the Geneclamp and then to the membrane patch. A classical P/4 protocol of pulses was used to remove residual leak current, if any, and residual capacitance artifact (Almers et al., 1983). The currents were further analysed off-line using WCP to calculate the ionic conductances and other parameters such as time constants. The currents and conductances were

standardized assuming that the surface of patched membrane was equal to the opening of the tip of the pipette. Some experiments were carried out in parallel to observe the effect of some of the compounds on the spontaneous beating rhythm of the cultured cells. In these cases the beating frequency was recorded using an automated system based on a specially designed system connected to a computer *via* the serial port. Visual observation was used to check the method.

Patch clamp solutions

As the stability and the resistance of the seals were lower in the culture medium, the patch experiments were performed after replacing the culture medium with a modified sterilized and filtered (0.22 μm) seawater containing the following major ions: NaCl, 456 mmol l^{-1}; KCl, 9.7 mmol l^{-1}; $CaCl_2$, 11 mmol l^{-1}; $MgCl_2$, 55.6 mmol l^{-1}. This medium contained neither proteins nor antibiotics, but the ions were present at the same concentration as in the culture medium. It was equilibrated with air; the pH was 7.3 at room temperature. The medium was changed more than 3 h before the experiments in order to allow stabilization of the characteristics of the cells. If used, channel blockers were added to the medium bathing the cells and to the medium filling the pipette: tetraethyl ammonium chloride (TEA), tetrodotoxin (TTX), charybdotoxin (CHTX) and verapamil at the concentrations stated in the text or in the figure legends. Effects of TTX, CHTX and verapamil were rapid, so recordings were made 3–10 min following their addition. For TEA, recordings were only made after 30 min, which was the incubation time required to achieve a maximum and steady inhibition. All the chemicals were purchased from Sigma (L'Isle-d'Abeau, France).

Statistical analysis

Results are given as means ± S.E.M. The normality of the distributions was verified using the Shapiro–Wilk test; mean values were compared by using parametric (Student's *t*-test or Aspin–Welch test) or non-parametric (Mann–Whitney) tests. Values were considered to be significantly different when $P<0.05$.

Results

Identification of ionic currents

Preliminary studies using intracellular micro-electrodes were performed to determine the resting membrane potential of the cultured cells, isolated from the atrium of the oyster heart. The mean value was –45.8±2.5 mV (N=35). This value does not significantly differ from the mean value previously found in *Pecten maximus*, under similar cell type and culture conditions (– 46.2±4.2 mV; N=12; J.-P.P., unpublished results), and is also in agreement with the value reported by Shigeto (1970) in *Crassostrea* myocardium (–45 mV). The distribution of the values was unimodal, indicating that the studied cells represented a homogenous population. Recordings of ionic currents were performed using a macro-patch clamp technique. A typical recording performed under control conditions and showing the summation of several currents is shown in Fig. 1A. The identification of the different underlying currents was then carried out by using specific inhibitors.

Potassium currents

Delayed potassium current

In fibres treated with tetrodotoxin (TTX) and verapamil to block Na^+ and Ca^{++} inward currents, respectively, outward currents were observed in all cells when depolarizing pulses were applied to the patch (Fig. 1B). These currents appeared to be composed of at least two different currents involving two different types of channels. A fast-activated, rapidly inactivated outward current was observed in most cells, superimposed by a slow-activated, sustained current. By using 10^{-5} mol l^{-1} of 4-aminopyridine (4-AP), the first current that showed fast activation and inactivation could be suppressed. Only the slow-activating, non-inactivating current remained. This current was blocked by 40 mmol l^{-1} of tetraethyl ammonium (TEA), a well known potassium channel blocker, and was identified as a classical delayed outward rectified potassium current (I_K), which is observed in most excitable cells (Fig. 1C). The current–voltage relationship (*I–V* curve) showed the usual pattern (Fig. 1D) with a strong rectification. The maximum conductance was calculated from the slope of the linear part of the *I–V* relationship and normalized in mS cm^{-2}, as described in Materials and methods. Under normal conditions, the mean value of the maximum conductance was 11.47±0.90 mS cm^{-2} (N=8). With 30 mmol l^{-1} of TEA in the bath, the conductance was reduced to 2.54±0.84 mS cm^{-2} (N=6), which gives a strong inhibition (78%). An order of magnitude of the time constant of the activation of the I_K current in control conditions can be determined by fitting the current obtained with the 150 mV depolarizing pulse onto a single exponential curve. In these conditions, the computed value was 53.01±0.40 ms (N=8).

Fast inactivating current

In well-polarized cells, or when the depolarizing test pulse was preceded by a hyperpolarizing pre-pulse (–40 mV, 120 ms), a transient outward current could be observed (see Fig. 1A,B). Characterization of the transient current was carried out in medium containing 30 mmol l^{-1} TEA, which strongly inhibited the delayed potassium current (see above) but only slightly reduced the amplitude of the transient current (Fig. 2A). It should be noticed that higher concentrations of TEA in the medium (40 mmol l^{-1}; 30 min incubation) had a significant inhibitory effect on the transient current, but this current was more selectively blocked by 4-AP at a concentration of 10^{-5} mol l^{-1} in the pipette (Fig. 2B), and was also inactivated by 120 ms depolarizing pulses applied immediately before the test pulse (Fig. 2C). Application of depolarizing steps regularly spaced from –30 to +110 mV allowed the plotting of the inactivation curve showed in Fig. 2D. From this curve it can be determined that a depolarizing

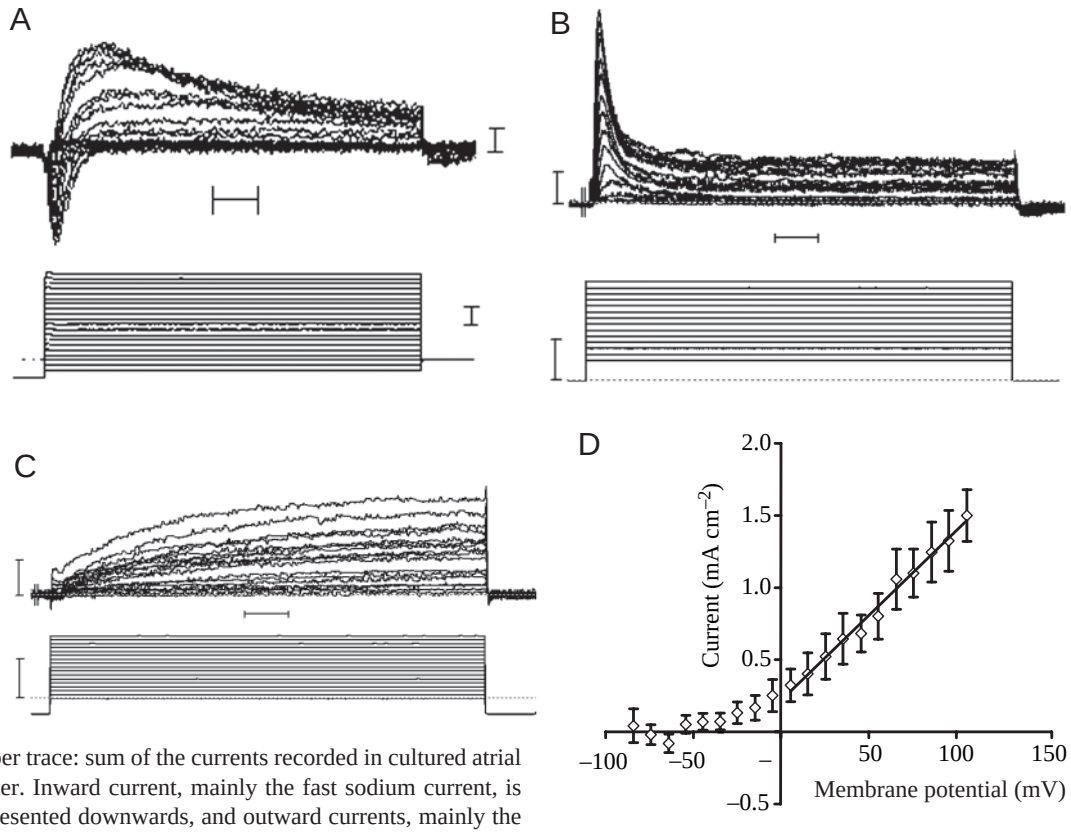

Fig. 1. (A) Upper trace: sum of the currents recorded in cultured atrial cells from oyster. Inward current, mainly the fast sodium current, is classically represented downwards, and outward currents, mainly the potassium currents, upwards. Lower trace: voltage steps imposed to the membrane. Depolarizing steps are upwards. Horizontal bar, 1 ms; vertical bars, 0.1 nA (top) and 40 mV (bottom). (B) Upper trace: sum of the fast inactivating and of the delayed potassium current. Lower trace: voltage steps applied to the membrane. Horizontal bar, 10 ms; vertical bars, 0.1 nA (top) and 60 mV (bottom). (C) Upper trace: delayed potassium current recorded after inhibition of the fast inactivating potassium current by 4-aminopyridine. Lower trace: voltage steps applied to the membrane. Horizontal bar, 10 ms; vertical bars, 0.1 nA (top) and 60 mV (bottom). (D) Current–voltage relationship of the delayed potassium current. The maximum conductance is calculated from the slope of the linear part.

voltage averaging +53 mV above the resting potential induced a 50% inhibition of the normalized current. This voltage corresponds to a membrane potential of about +7 mV, according to the mean value of the resting membrane potential.

The *I–V* curve corresponding to the fast I_K current is depicted in Fig. 3. It also shows a strong rectification; the maximum conductance was 30.33±3.73 mS cm^{-2} (*N*=7). By performing a regression according to Hodgkin–Huxley type equations, the time constant of activation (τ_m; 0.484±0.027 ms) and the time constant of inactivation (τ_h; 2.160±0.051 ms) could be determined for a 150 mV depolarizing pulse.

Maxi potassium current

In most cells, but not in all the cells, a large outward current was observed with strong depolarizing pulses (Fig. 4A). The *I–V* curve gave a maximum conductance of 387.13±31.42 mS cm^{-2} (*N*=6). This current was reduced in verapamil-treated cells and suppressed by 10^{-5} mol l^{-1} of charybdotoxin (CHTX); it was then attributed to a calcium activated potassium channel (K_{Ca}). This current was mainly elicited by strong depolarisations, but its large conductance suggested that, even with moderate depolarisations (see Fig. 4A), its intensity was not negligible. Hence, this current could play a role in the stabilization of membrane potential and in the reduction of the spontaneous beating rate. This hypothesis was in agreement with the fact that, in spontaneously beating cultured cells, inhibition of this current by CHTX increases the beating rate in a dose-dependent way, as shown in Fig. 4B.

Sodium current

A fast transient inward current was observed in some cells. This current was inhibited by 10^{-6} mol l^{-1} of TTX (tetrodotoxin) and was identified as the classical voltage-gated sodium current. An example of this current is reported in Fig. 5. Note that it was not observed in all the cells. The sodium current was only triggered if a hyperpolarizing pulse (amplitude –60 mV, duration 120 ms) was applied just before the test pulse, and suggests that this current should be inactivated at the normal membrane potential of the cultured cells and cannot be involved in the triggering of the spontaneous beatings. Calculated from the *I–V* curve, the maximum conductance was 20.54±0.56 mS cm^{-2} (*N*=8). τ_m and τ_h of the sodium current were calculated according to the

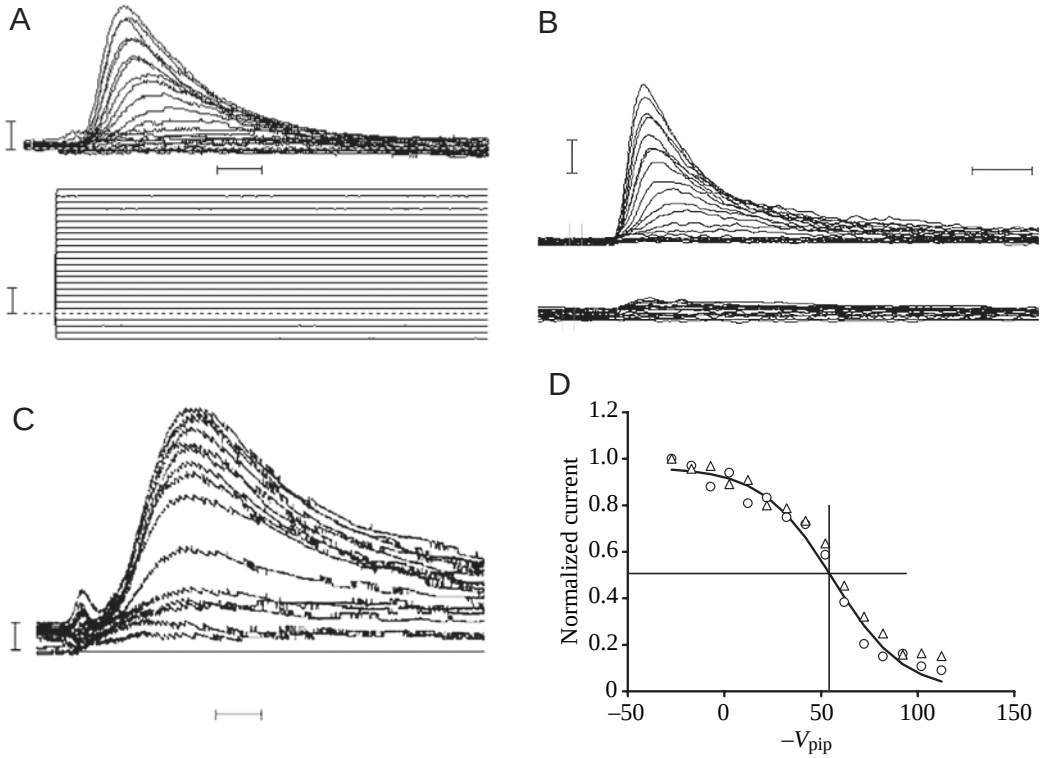

Fig. 2. (A) Upper trace: fast inactivating potassium current recorded after inhibition of the delayed potassium current by tetraethyl ammonium. Lower trace: voltage steps applied to the membrane. Horizontal bar, 1 ms; vertical bars, 0.1 nA (top) and 40 mV (bottom). (B) Upper trace: fast inactivating potassium current recorded after inhibition of the delayed potassium current by tetraethyl ammonium. Lower trace: inhibition of the fast inactivating potassium current by 4-amino pyridine. Horizontal bar, 1 ms, vertical bar, 0.1 nA. (C) Inhibition of the fast inactivating potassium current by depolarizing pulses applied to the membrane before the test pulse. Horizontal bar, 1 ms, vertical bar, 0.1 nA. (D) Inhibition curve of the I_K fast current by depolarizing pulses. Experimental points are fitted onto a Boltzman-type equation. Vertical: normalized current expressed as measured current/maximum measured current. Horizontal: depolarizing pulse expressed as minus the pipette voltage V_{pip}.

Hodgkin–Huxley equation using the curve-fitting option available in the WCP software; the values obtained were 0.330±0.011 ms and 1.110±0.31 ms, respectively.

Calcium currents

In cells treated with 40 mmol l^{-1} TEA, an inward current, which was inhibited by 10^{-5} mol l^{-1} verapamil or 5–10^{-3} mol l^{-1} cobalt ions, was identified as a calcium inward current (Fig. 6A). The activation of this current was rather slow, with a time constant averaging 44.01±0.17 ms; no inactivation was seen with relatively short duration pulses (100–120 ms). This slow inward current was increased by serotonin (5HT) as shown in Fig. 6B. The corresponding conductance was similarly increased; control conductance, 6.82±1.44 mS cm^{-2} (N=16), conductance with 10^{-6} mol l^{-1} of 5HT, 14.09±0.25 mS cm^{-2} (N=10). The dose-dependant increase induced by 5HT was reversed by verapamil (10^{-6} mol l^{-1}); the conductance was reduced to a value not differing from control (6.05±0.84 mS cm^{-2}; N=6). τ_m was also decreased. In addition it was noticed that 5HT could induce a

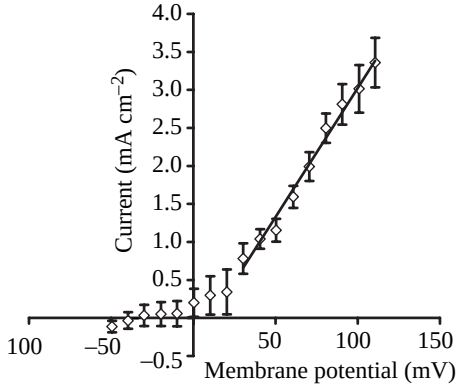

Fig. 3. Current–voltage relationship of the fast inactivating potassium current. The maximum conductance is calculated from the slope of the linear part (see text).

spontaneous activity in some quiescent cells. Experiments in cultured, spontaneously beating cells (4–6 days old cultures) showed that 5HT induced a dose-dependent increase in the

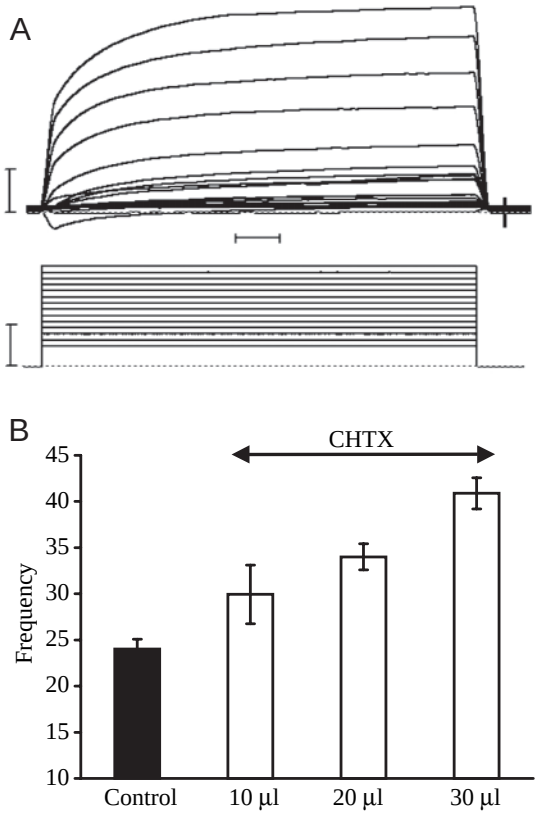

Fig. 4. (A) Upper trace: maxi potassium current recorded after inhibition of the fast inactivating potassium current by 4-aminopyridine. Lower trace: voltage steps applied to the membrane. Horizontal bar, 10 ms; vertical bars, 0.2 nA (top) and 60 mV (bottom). (B) Effect of charybdotoxin (CHTX) on the spontaneous beating rate of atrial cultured cells from oyster. Vertical: beating frequency. Horizontal: CHTX addition in Petri dishes corresponding to a final concentration of: 5×10^{-6}, 10^{-5}, 1.5×10^{-6} mol l^{-1}. All the CHTX values are significantly different from the control.

beating rate of the cells, as shown in Fig. 7; 50% of the maximum effect was obtained with 10^{-9} mol l^{-1}. Concentrations of 5HT higher than 10^{-6} mol l^{-1} induced very strong contractions, which ruptured the cellular networks and caused detaching of the cells from the bottom of the dishes. The effect of 5HT on the beating rate was reversed by verapamil (10^{-6} mol l^{-1}), which induced a return to a frequency not different from control (16.00±0.97 beats min^{-1} *vs* 16.11±1.63 beats min^{-1}; N=16 and N=8, respectively). Higher concentrations of verapamil (10^{-5} mol l^{-1} or higher) largely impaired the spontaneous contractile activity. The slow inward current was also inhibited by cobalt ions, as shown in Fig. 6C. In some cells, when delivering a 80 mV hyperpolarizing pulse to the membrane before the test pulse, a transient inward current was observed, with τ_h ~41.50±2.50 ms (Fig. 8).

Cryopreserved cells

In order to standardize the culture and then to facilitate the use of the cultured cells, e.g. for carrying out toxicological experiments, attempts were made to cryopreserve the cells. The

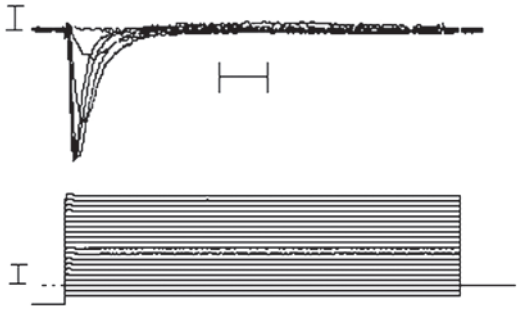

Fig. 5. Example of a recording of the fast sodium current after tetraethyl ammonium and 4-aminopyridine treatment. Horizontal bar, 1 ms; vertical bars, 0.1 nA (top) and 40 mV (bottom).

technique modified from Le Marrec et al. (1998) and briefly described in the Materials and methods, was used to freeze isolated cells. The frozen cells could be stored for long periods in liquid nitrogen and then thawed and cultured just like freshly isolated cells. The percentage of viable cells, evaluated after thawing by a Trypan Blue exclusion test, was better than 75%. This is lower than the percentage of viable unfrozen cells (95%) but higher than viable thawed cells from *Pecten maximus* (P. Fritayre, unpublished), which represented only 30%. The cells, cultured after thawing, were adherent to the bottom of the plastic dishes in 5–6 days; they were spontaneously beating at 12 days and they formed contractile networks after 10–15 days, instead of, respectively, 2 days, 5 days and 8 days for the unfrozen cells. It can be assumed that the first stages of culture are slower for thawed cells than for

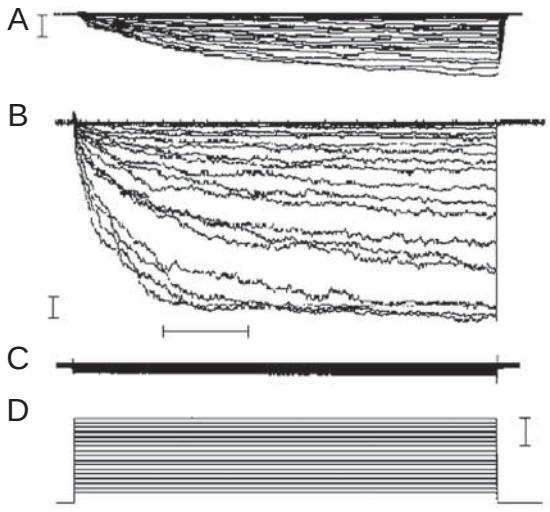

Fig. 6. (A) Example of a recording of the calcium inward current after treatment with 40 mmol l^{-1} tetraethyl ammonium chloride, 10^{-6} mol l^{-1} TTX and 40 mmol l^{-1} 4-aminopyridine treatment. (B) Effect of the addition of 10^{-5} mol l^{-1} of serotonin (5HT) on the calcium current. (C) Blockade of the calcium current after addition of cobalt ions in the medium bathing the cells and in the pipette. (D) Imposed voltages. Vertical bars, 0.1 nA (A–C) and 60 mV (D); horizontal bar, 10 ms.

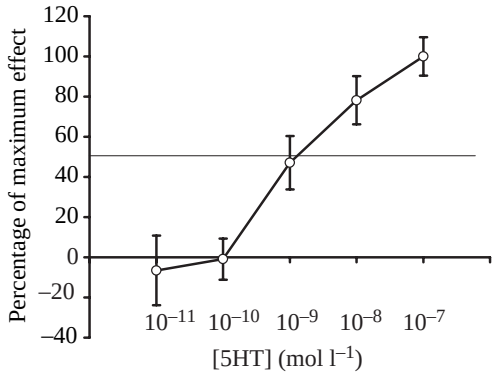

Fig. 7. Effect of serotonin (5HT) on the spontaneous beating rate of *in vitro* atrial cultured cells. Vertical: percent of the maximum effect. Horizontal: serotonin corresponding to a final concentration ranging from of 10^{-11} to 10^{-7} mol l^{-1}. A significant increase ($P<0.05$) was observed at concentrations of 5HT equal or higher than 10^{-9} mol l^{-1}. Values obtained with concentrations equal or higher than 10^{-6} mol l^{-1} are not plotted because the beatings became erratic, and the cells were detaching themselves from the plastic bottom of the Petri dishes, showing a toxic effect at such concentrations.

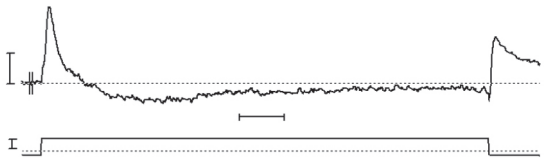

Fig. 8. Example of a transient inward calcium current following a fast outward potassium current. The cell was treated with tetraethyl ammonium chloride. Vertical bars, 0.1 nA (top) and 40 mV (bottom); horizontal bar, 10 ms.

unfrozen cells. However after the first 2 weeks, the cultures appeared very similar and the spontaneous beating rate was not significantly different; 29.4±1.2 beats min^{-1} in the normally cultured cells *vs* 27±0.82 beats min^{-1} in the thawed cells (N=8). At this stage, measurements were made to determine if freezing/thawing induced significant and durable modifications in the major ionic currents. Only slight modifications were recorded; the delayed potassium conductance was 9.44±0.02 mS cm^{-2} and the calcium conductance was 6.30±0.71 mS cm^{-2}. These values were slightly lower but not significantly ($P>0.05$) different from the values found in normally cultured cells. Similarly, the fast potassium conductance was not modified. Only the sodium current could not be clearly identified in thawed cells, but it was not present in all the normally cultured cells in any event. In addition, the effects of blockers are not modified. After 2 weeks, no significant difference was found between the two types of cultured cells.

Discussion

In a previous paper, inward and outward currents were observed in cultured cells isolated from the atrium of oyster heart (Pennec et al., 2002), but precise identification and characterization of the different underlying currents were not carried out. These preliminary results indicated that the values of the major ionic currents, inward sodium and calcium currents and delayed potassium current, appeared fairly constant when they were measured shortly after seeding and during 25 days of culture; only a slight, not significant, decrease in inward current was observed. Thus the dissociation process and culture induced no major modification in the structure of these channels. However, changes in protein expression, including ion channels, cannot be excluded in relatively long-term culture conditions. In addition, network formation could modify intercellular coupling. Minor and reversible membrane alterations due to the dissociation process could explain why freshly isolated cells showed no spontaneous beating and had also a reduced sensitivity to catecholamines. In the previous study, the outward, fast-inactivating potassium current was not measured and its variations during the duration of the culture were not quantified. In the present study this current was never observed in freshly isolated, non-beating cells.

It was necessary to characterize the ion channels in steady-state conditions and in fully functional cells. As it was technically not possible to perform patch-clamp experiments on cells *in vivo*, and as the cells were forming contractile clusters that were not suitable for patch-clamp studies after 4 weeks of culture, the present study focused on the characterization of voltage-dependent ion currents underlying the electrophysiological properties of cultured atrial cardiomyocytes of the oyster within the culture period giving steady electrophysiological properties (5–25 days after seeding).

The following currents were identified under these conditions:

(1) A non-inactivating or delayed outward rectifying potassium current, very similar to that observed in many other cells, e.g. neurons or in cardiomyocytes isolated from molluscs (Curtis et al., 1999; Yeoman and Benjamin, 1999). This current is TEA-sensitive but the sensitivity can vary according to the tissue or species. In oyster atrial cells, the sensitivity appears to be higher than in mussel cardiac cells: TEA (20×10^{-3} mol l^{-1}) induced a significant inhibition and at 30×10^{-3} mol l^{-1}, a drastic (78%) inhibition of the potassium current in the oyster cells, whereas 30×10^{-3} mol l^{-1} of TEA induced only a 27% inhibition in the mussel cells (Curtis et al., 1999). The range is closer to that observed in mammals, where a total inhibition is observed with TEA at a concentration of 50×10^{-3} mol l^{-1}. One explanation for the greater sensitivity of oyster cells compared to mussel cells could be that, as in the present study, the cells were incubated in the TEA-containing medium for 30 min before recording, thus allowing the penetration of some amount of TEA into the cells and increasing the efficiency of the blockage. However, the possibility of a specific difference cannot be excluded.

(2) A fast activated, spontaneously inactivated potassium current, which has been identified in many cardiac cells in

invertebrates (Curtis et al., 1999; Yeoman and Benjamin, 1999) and vertebrates, including mammals (Coraboeuf and Carmeliet, 1982; Giles and van Ginneken, 1985). This current looks like the A-current previously identified in mollusc neurons (Connor and Stevens, 1971) or the I_{to} current identified in mammalian heart cells (Greenstein et al., 2000) and involved in the rapid initial phase of action potential repolarization. More recently, such a current was identified in mussel heart cells (Curtis et al., 1999). This current is partly responsible for the initial repolarization process: the 'notch' in mammalian ventricular action potential. It was not present in pacemaker cells from the sino-atrial node in mammals (Irisawa et al., 1993) but was identified in isolated spontaneously beating cells of *Lymnea* (Yeoman and Benjamin, 1999), where it was involved in the determination of the beating rate. According to this hypothesis, our experiments demonstrate that 4-AP induced an increase in the spontaneous beating rate of the cultured oyster cells by inhibiting this fast inactivating K^+ current. This current appears less sensitive to TEA than the delayed rectifying K^+ current, in contrast to the observations in mussel (Curtis et al., 1999), where the sensitivity of both K^+ currents is in the same range. In oyster, it was not observed in medium containing high concentrations of TEA (60 mmol l^{-1}) but as it was not observed in all the cells, even in normal medium, it is difficult to be sure if there was a total inhibition. The reason why it was lacking in some cells could be either the tissue origin or, more certainly, a different physiological state; all the cells were isolated from the atrium but they were not all spontaneously beating. In addition, this spontaneous activity appeared only after 4–5 days of culture, depending on the temperature, and in parallel this current was identified neither in freshly isolated cells nor during the first days of culture. In addition, this current was inactivated by application of preliminary depolarizing pulses as shown by the inactivation curve, so in cells showing a low membrane potential, this current may be simply inactivated. Then it could be hypothesized that this current is characteristic for well-polarized, differentiated cells with fully functional channels. It could be involved in the modulation of the spontaneous activity even if it was not responsible for the triggering of this activity because of its repolarizing effect.

(3) A maxi potassium current, mainly activated with large depolarizing pulses, and largely sensitive to calcium influx, as shown by the inhibiting effect of verapamil. This current should be a calcium-activated potassium current (K_{Ca}), as it is blocked by CHTX. It was not observed in mussel cells (Curtis et al., 1999) but it is present in many species of vertebrates and in molluscs like the squid (Ödblom et al., 2000). This channel could be activated by small depolarisations and, because of its large conductance, could also be involved in the control of the spontaneous activity of these cells by stabilizing the membrane potential. The increase observed in spontaneous beating rate of the cultured cells following the inhibition of this current by CHTX is in agreement with such a hypothesis.

An inward rectifying potassium channel was not found in oyster atrial cells. This parallels findings on cardiac cells of the mussel (Curtis et al., 1999).

(4) A rapid sodium inward current. This current appears to be very similar to the fast sodium current observed in many excitable cells, and especially cardiac cells, in molluscs such as mussel (Curtis et al., 1999), and vertebrates. The difference could be a slower activation and a slower inactivation when compared to the values reported in mammals (Ruff, 1992), which are about the half of those determined here. In order to better evaluate the difference, the fast sodium current was measured in isolated rat muscle fibres using the same experimental setup and at the same temperature. Values of 0.168±0.046 ms and 0.207±0.019 ms (N=8) were obtained for τ_m and τ_h, respectively, in rat muscle fibres. These values were one half and one fifth, respectively, of the values found in the oyster atrial cells. The maximum value of the conductance measured in the rat fibres was 62.0±1.0 ms cm^{-2} (N=8); this value is about three times higher than the value found in the atrial cells. There are two possible explanations for this: either the sodium channels are less abundant in these atrial cells than in the rat muscle fibres or, but not exclusively, these channels are of a different subtype. The differences in activation and inactivation time constants at similar temperature and potentials favoured this second possibility.

(5) A calcium inward current. Such a current is well known in cardiac cells that demonstrate contractility. Evidence that the calcium current indicated in our experiments is flowing through L-type calcium channels includes: (i) it showed no noticeable inactivation within the duration of our test pulses, and was inhibited by verapamil and cobalt ions; (ii) it can also be strongly reduced by cadmium (Pennec et al., 2002). A more precise investigation should be carried out to assess whether both types (L and T) of calcium channels are present in the cells. However, when a strong hyperpolarization (–60 mV to –70 mV) was applied to the membrane before the test pulse a transient inward current was observed in some cells, suggesting that transient calcium channels exist in the membrane but are normally inactivated by the relatively low resting potential of the cultured cells (–45.8 mV). 5HT increased both the calcium conductance and the beating rate of the cultured cells *in vitro*. Moreover, 5HT triggered a spontaneous beating activity in quiescent cells. These effects were reversed by verapamil, showing that calcium channels are involved in the determination of the spontaneous beating rate as reported by Yeoman et al. (1999) in the ventricular muscle cells of the *Lymnaea* heart. Pennec et al. (2002) previously showed that the slow inward current could also be increased by epinephrine, and that this effect was reversed by high doses of propranolol, thus demonstrating that the heart rate could also be modulated by beta-adrenergic agonists. However, the effects of epinephrine were not highly dose-dependent and were observed at relatively high concentrations (10^{-6} mol l^{-1}), suggesting that epinephrine is not the prominent catecholamine in the modulation of the cardiac rhythm. Serotonin is another possibility as it is released by neurons and could then play a role in the control of the heart rate of the oyster *in vivo* (Lee, 1993; Kioaki Kuwasawa and Hill,

1997). The present results, showing that the concentration giving 50% of the maximum effect was as low as 10^{-9} mol l^{-1}, are in agreement with such a hypothesis.

Besides of its normal regulation, this current is inhibited by some toxic chemicals such as tributyltin (TBT) and cadmium, which are found in moderately polluted seawater at concentrations ranging from 10^{-12} to 10^{-10} mol l^{-1} (Shim et al., 1998; Thornton, 1992). These chemicals induced a decrease in both inward and outward currents, depending on the concentration and on the time of exposure, as previously reported (Pennec et al., 2002). New measurements gave an inhibition of 46% for the sodium conductance, 47% for the calcium conductance and only 33% for the outward rectified potassium conductance with a concentration of 10^{-9} mol l^{-1} of cadmium in the medium (J.-P.P., unpublished results). Significant inhibition was observed with concentrations lower than 10^{-9} mol l^{-1} (i.e. 10^{-12} mol l^{-1} after 12 days of exposure), revealing the sensitivity of this bioassay.

In addition to voltage-operated channels, acetylcholine (Ach) increases the global potassium conductance (Pennec et al., 2002), showing that these cells also have Ach-activated potassium channels. As the effect of Ach was blocked by atropine, it was assumed that the cultured cells had functional muscarinic M2-like receptors.

Globally, our results agree well with those reported for freshly isolated mussel cardiac cells (Curtis et al., 1999), despite the fact that our experiments were performed on long-term cultured cells that showed spontaneous contractile activity. It can be suggested that in freshly isolated cells, ion channels could be more or less altered by the enzymatic dissociation while they are fully functional after a few days of culture. In agreement with this, cells were beating after only a few days of culture and epinephrine or acetylcholine had a minor or even no effect during these very first days of culture, which could also explain the differences in the identified currents that were noticed: the increased sensitivity of the delayed potassium current to TEA, the existence of a calcium-activated potassium current and the existence in some cells of T-type calcium currents, which were not seen in mussel. These currents were described in *Lymaea* ventricular cells (Yeoman et al., 1999). We suggest that both the maxi potassium current and the fast inactivating potassium current are involved in the modulation of spontaneous beating rate along with the antagonistic calcium current. The potassium currents reduce the spontaneous beating rate while the calcium current increases it. The transient inward calcium current could be related to the triggering of the spontaneous activity, as suggested by Yeoman et al. (1999) but as it was not seen in all the cells of the present study we hypothesize that it is limited to the pacemaker cells.

The technique of cryopreservation could be very useful for bioassays: cells can be stored for a long time before use without any significant alteration in their electrophysiological properties. Associated with long-term culture, it provides the possibility of testing cardioactive compounds in acute or in chronic conditions.

To summarize, the present work shows that the atrial cells isolated from oyster heart present ionic currents very similar to the currents described in homologous cells of many molluscs or vertebrates. This gives fundamental information about the electrophysiological properties of these cells. Despite a slightly slower development of the contractile networks, the culture of cryopreserved cells is possible without any major alteration of their electrophysiological properties, thus increasing the versatility and the interest of the model. Hence freshly isolated and cryopreserved cells can be used as a model for the study of acute and chronic effects of natural or exogenous marine molecules. Preliminary studies with freshly isolated cells on the toxicity of marine pollutants such as cadmium and tributyltin showed that the sensivity of this assay was several orders of magnitude higher than the sensitivity of the usual *in vivo* experiments (Pennec et al., 2002).

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References

Almers, W., Stanfield, P. R. and Stümer, W. (1983). Slow changes in currents through sodium channels in frog muscle membrane. *J. Physiol.* **339**, 253-271.

Brezden, B. L. and Gardner, D. R. (1992). A review of the electrophysiological, pharmacological and single channel properties of heart ventricle muscle cells in the snail *Lymnaea stagnalis*. *Experientia* **48**, 841-852.

Chow, R. H. (1991). Cadmium block of squid calcium current. *J. Gen. Physiol.* **98**, 751-770.

Connor, J. A. and Stevens, C. F. (1971). Voltage clamp studies of a transient outward membrane current in gastropod neural somata. *J. Physiol. Lond.* **213**, 21-30.

Coraboeuf, E. and Carmeliet, E. (1982). Existence of two transient outward currents in sheep cardiac Purkinje fibers. *Pflügers Arch.* **392**, 352-359.

Curtis, T. M., Delpledge, M. H. and Willamson, R. (1999). Voltage-activated currents in cardiac myocytes of the blue mussel, *Mytilus edulis*. *Comp. Biochem. Physiol.* **124**, 231-241.

Giles, W. R. and van Ginneken, A. C. (1985). A transient outward current in isolated cells from the crista terminalis of rabbit heart. *J. Physiol.* **368**, 243-264.

Greenberg, M. J., Roop, T. and Painter, D. S. (1980). The relative contributions of the receptors and cholinesterases to the effects of acetylcholine on the hearts of bivalve molluscs. *Gen. Pharmacol.* **11**, 65-74.

Greenstein, J. L., Wu, R., Po, S., Tomaselli, G. F. and Winslow, R. L. (2000). Role of the calcium independent transient outward current I_{to1} in shaping action potential morphology and duration. *Circ. Res.* **87**, 1026-1033.

Irisawa, H., Brown, H. F. and Giles, W. R. (1993). Cardiac pacemaking in the sinoatrial node. *Physiol. Rev.* **73**, 197-227.

Kioaki Kuwasawa, K. and Hill, R. B. (1997). Evidence for cholinergic inhibitory and serotoninergic excitatory neuromuscular transmission in the heart of the bivalve *Mercenaria mercenaria*. *J. Exp. Biol.* **200**, 2123-2135.

Lee, C. M., Lin, J. T. and Tsai, T. S. (1993). Effects of neuroactive agents on the isolated heart activities of marine bivalve *Meretrix lusoria*. *Chinese J. Physiol.* **36**, 165-170.

Le Marrec-Croq, F., Fritayre, P., Chesné, C., Guillouzo, A. and Dorange, G. (1998). Cryopreservation of *Pecten maximus* heart cells. *Cryobiol.* **37**, 200-206.

Le Marrec-Croq, F., Glaise, D., Guguen-Guillouzo, C., Chesné, C.,

Guillouzo, A., Boulo, V. and Dorange, G. (1999). Primary cultures of heart cells from the scallop *Pecten maximus* (mollusca-bivalvia). *In Vitro Cell. Dev. Biol. Animal* **35**, 289-295.

Malecot, C. O. and Duval, A. (1992). A macro cell-attached patch-clamp study of the properties of the Na current in the vicinity of the motor endplate region of frog single interosseal skeletal muscle fibres. *Pflügers Arch.* **420**, 231-238.

Ödblom, M. and Williamson, R. (1995). Ionic currents in branchial heart myocytes of squid, *Alloteuthis subulata* and *Loligo forbesii*. *J. Physiol. Lond.* **489P**, 66P.

Ödblom, M. P., Williamson, R. and Jones, M. B. (2000). Ionic currents in cardiac myocytes of squid, *Alloteuthis subulata*. *J. Comp. Physiol. B* **170**, 11-20.

Peirera Ferreira, R. P. and Salomão, L. C. (2000). The ionic basis of cardiac activity in the bivalve mollusc *Perna perna*. *J. Exp. Mar. Biol. Ecol.* **249**, 1-12.

Pennec, J. P., Gallet, M., Gioux, M. and Dorange, G. (2002). Cell culture of bivalves: tool for the sudy of environnemental stressors. *Cell. Mol. Biol.* **48**, 351-358.

Ruff, R. L. (1992). Na current density at and away from end plates on rat fast and slow-twitch skeletal muscle fibers. *J. Physiol.* **262**, C229-C234.

Shigeto, N. (1970). Excitatory and inhibitory actions of acetylcholine on hearts of oyster and mussel. *Am. J. Physiol.* **218**, 1773-1779.

Shim, W. J., Oh, J. R., Kahng, S. H., Shim, J. H. and Lee, S. H. (1998). Accumulation of tributyl- and triphenyl compounds in Pacific oyster, *Crassostrea gigas*, from the Chinhae Bay system, Korea. *Arch. Environ. Contam. Toxicol.* **35**, 41-47.

Thornton, I. (1992). Sources and pathways of cadmium in the environment. *IARC Sci. Pub.* **71**, 33-42.

Wilkens, L. A. (1972). Electrophysiological studies on the heart of the bivalve mollusc, *Modiolus Demissus*. II. Ionic basis of the action potential. *J. Exp. Biol.* **56**, 293-310.

Yeoman, M. S. and Benjamin, P. R. (1999). Two types of voltages-gated K (+) currents in dissociated heart ventricular muscle cells of the snail *Lymmnaea stagnalis*. *Neurophysiol.* **82**, 2415-2427.

Yeoman, M. S., Brezden, B. L. and Benjamin, P. R. (1999). LVA and HVA Ca^{++} currents in ventricular muscle cell of the *Lymnaea* heart. *J. Neurophysiol.* **82**, 2428-2440.