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EFFECTS OF INTERNAL FREE CALCIUM UPON THE SODIUM AND CALCIUM CHANNELS IN THE TUNICATE EGG ANALYSED BY THE INTERNAL PERFUSION TECHNIQUE

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SUMMARY

1. The unfertilized egg of the tunicate, *Halocynthia roretzi*, was intracellularly perfused with various solutions.

2. The perfusion apparatus consisted of lower and upper compartments which were connected by a small glass funnel. A denuded egg cell without chorion was dropped into the funnel and brought into close contact with the glass wall of the funnel. The membrane of the egg faced to the lower compartment was ruptured by a slight difference of hydrostatic pressure and the inside of the egg was perfused with the internal solution flowing through the lower compartment. The current across the upper membrane was analysed by voltage-clamp technique.

3. The egg cell in contact with 400 mM-Na external solution and perfused intracellularly with 400 mM-Na for 30 min showed a relatively low Na reversal potential, +6 mV, in comparison with +60 mV in the intact egg in standard artificial sea water. The exchange efficiency was monitored by observing the shift of Na reversal potential during perfusion with high Na internal perfusate.

4. The internal perfusate containing F^- ions stabilized the egg membrane and kept the excitability for 1–2 hr during the intracellular perfusion. With the internal F^- perfusate the intracellular cationic content was changed to 400 mM-Na, K, Rb or Cs (external solution of 400 mM-Na) and permeability ratios of the egg Na channel were estimated as $P_{Na}:P_K:P_{Rb}:P_{Cs} = 1{\cdot}0:0{\cdot}14:0{\cdot}05:0{\cdot}04$.

5. The internal F^- perfusate abolished Ca current which was consistently observed in the intact egg, while the internal Cl^- perfusate kept both Na and Ca current as in the intact egg. However with the internal Cl^- perfusate the egg cell could not be kept in good condition more than 20–30 min.

6. The effects of intracellular free Ca ions upon the egg Na and Ca channels were analysed by using Ca ion-buffered internal Cl^- and high Na perfusate. The results showed that internal Ca ions above 10^{-6} M reduced the Ca current and enhanced the Na current at the same time. In the range between 10^{-5} and 10^{-4} M the Ca current became half of the control obtained with zero free Ca perfusate while the Na

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conductance at the zero current level doubled. The internal Ca ions above one mM seemed to abolish the Ca current and to reduce the Na current as well. The reciprocal effect of intracellular Ca ions upon the egg Na and Ca channels was demonstrated in the concentration range from 10^{-6} to 10^{-3} M.

INTRODUCTION

Previous studies on the development of the excitability in the embryonic membrane of the tunicate have shown that the unfertilized egg cell membrane is excitable, having Na, Ca, delayed K, and anomalous K channels which are identical with those found in other excitable membrane (Miyazaki, Takahashi & Tsuda, 1974; Okamoto, Takahashi & Yoshii, 1976*a*). In the differentiated tadpole larva of the tunicate, Ca channels were segregated in the muscle membranes, while Na channels were assumed to be accumulated in the presumptive nerve cells (Miyazaki, Takahashi & Tsuda, 1972). Therefore, it is suggested that there are factors inside the egg cell which regulate the distribution of ionic channels on the embryonic membrane.

The importance of free Ca during development and differentiation has long been suggested, especially at the time of fertilization and cleavage (Stainhardt & Epél, 1974). Ca ionophore, which makes the membrane permeable to Ca, has been demonstrated to induce artificial parthenogenesis (Steinhardt, Epél, Carroll & Yanagimachi, 1974), and an increase in the intracellular free Ca at fertilization has been observed by injecting the photo-emitting protein 'aequorin' into the Medaka egg (Ridgway, Gilkey & Jaffe, 1977). The increased free Ca must have some function during development. Thus, it is questioned whether free Ca can play an important role in differentiation and development of the excitability of the membrane. Recently the internal perfusion technique has been reported successful in the isolated giant nerve cells of the mollusca (Kostyuk, Krishtal & Pidoplichko, 1975, 1977; Lee, Akaike & Brown, 1977). In the present study, this technique was applied to analyse the effect of internal Ca ions upon Na and Ca channels found in the tunicate egg cell membrane.

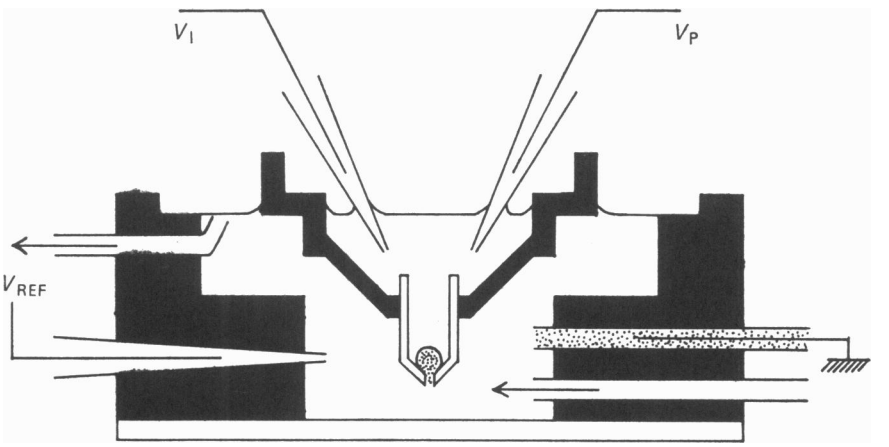
METHODS

Materials. The unfertilized eggs of the tunicate, *Halocynthia roretzi*, were used. The collection of the animals and the methods of spawning have been described previously (Miyazaki *et al.* 1974). The eggs used for the internal perfusion were prepared in sea water containing 0·5 % pronase at 10 °C for 2 hr in order to denude eggs of the follicular envelope and the chorion and to clean egg surface. After this enzymatic treatment, the denuded eggs were washed with seawater containing 0·5 % bovine serum albumin (BSA) to stop further digestion by the enzyme.

Perfusion apparatus. The perfusion apparatus consisted of upper and lower compartments. The two compartments were connected by a small funnel, as illustrated in Text fig. 1. The upper compartment was filled with artificial sea water (ASW) as an external solution and the lower compartment was continuously perfused with another solution as an internal solution. The flowing and cooling apparatus were similar to those described previously (Miyazaki *et al.* 1974). The funnel was made from a Pyrex micropipette (1·5 mm capillary). By heating the tapering portion with a microforge, the orifice of the cut end was made smooth and narrowed. The orifice diameter was 50–100 μm (average 80 μm), being approximately a quarter of the egg diameter (270 μm) (see Pl. 1). This small funnel was inserted into a hole at the bottom of the upper compartment and fixed with a dental wax to be watertight. A new funnel was used for each egg.

Setting an egg in the perfusion apparatus. To set an egg between the two compartments, the denuded egg was dropped in the small funnel and brought into contact with the glass wall inside.

To facilitate the contact, the hydrostatic pressure in the upper compartment was always kept at a few mm H_2O higher than that in the lower compartment, and the inside of the funnel was preliminarily soaked with 1 % protamine sulphate in distilled water. Protamine is a very basic protein which is capable of neutralizing negative surface charges on the glass wall. Contact of the egg with the glass wall was regarded as virtually complete when the resistance between two compartments became more than 1000 MΩ. The resistance was determined by measuring a potential drop produced by a constant current pulse applied through the small funnel. Following the establishment of contact, the lower part of membrane at the orifice was ruptured either spontaneously due to the hydrostatic difference or artificially by injecting a high frequency current through the funnel using the over compensation of high frequency response in the pre-amplifier. Immediately after rupture, the upper membrane was voltage-clamped at −90 mV level.



Text-fig. 1. Schematic representation of the internal perfusion apparatus for the tunicate egg. The apparatus consists of upper and lower Lucite chambers and a small glass funnel inserted at the bottom of the upper chamber. The lower chamber is set on a microscope stage and perfused with the internal perfusate. The upper chamber is attached to a frame connecting with a micromanipulator and is filled with the external solution. The electrodes labelled V_P, V_I and V_{REF} are the potential, current and reference electrodes, respectively. The potential and current electrodes are attached also to the frame for the upper chamber and the reference electrode is inserted into a hole made in the wall of the lower chamber. The wall of the lower chamber is also inserted by a ground electrode, inflow and outflow tubes and a thermister which is not illustrated. All gaps by these insertions are filled with wax. Light can pass through the bottom of the lower chamber and the glass funnel, allowing microscopical observation around the funnel.

Recordings. All measurements of the membrane current of the ruptured egg were done under the voltage-clamped condition. The potential and current electrodes were immersed in the upper compartment and the reference electrode was inserted in the lower compartment which was virtually grounded. All three electrodes were glass pipettes with large tip diameter (10–20 μm) filled with 3 M-KCl and connected by Ag–AgCl junctions to the input stages. Such large tips allowed the electrode resistances less than 1 KΩ. The zero-potential levels for the potential and current electrodes were adjusted by immersing all three electrodes in the internal solution just before each experiment. The potential drifts in the electrodes and in the recording systems were extremely small. Readjustment of the amplifier was not necessary during all succeeding experiments in a day. The liquid junction potential and tip potential between each electrode and the solution were always negligible, although the liquid junction potential between the internal and external solutions were usually in the range of 10–15 mV. The voltage-clamp circuit was the same as described previously (Okamoto *et al.* 1976*a*).

In the present experiment, the electrode resistances were almost negligible and open loop gain of the feed-back circuit could be increased up to more than 5000 without ringing at the beginning of the pulse. Thus, the rise time of a clamped voltage pulse was less than 0·2 msec. The series resistance due to the cytoplasm which remained in the funnel under the membrane may have disturbed the efficiency of the voltage-clamp. However, the actually measured series resistance was less than 10 kΩ because of the wide orifice of the funnel. The potential error due to the series resistance, therefore, was less than 0·5 mV even in the case of a maximum inward current of 50 nA. *The membrane potential* was described as the potential at the lower compartment minus the potential at the upper compartment. The current through the funnel was used as measure of the *membrane current*. The current from the lower to the upper compartment was defined as positive or outward current, and that in the opposite direction as negative or inward current. The holding potential was always set at −90 mV. The potential and current recordings in response to a step change in the membrane potential were memorized in two transient recorders (Kawasaki Electronica TM 1510, 8 bit × 1024 word) and later reproduced on a d.c. multi-channel pen recorder of a high speed and balanced type (Rikadenki, B-26 Mark II). The d.c. components of the potential and current recordings were also recorded directly on another d.c. multi-channel pen recorder.

TABLE 1. Composition of solutions

Internal solutions (mM)

	X^-	A^-	GEDTA	DPTA-OH	PIPES*	$Ca(OH)_2$	Glucose
A	400 Na	170 F^-	100	—	20	—	370
B	400 K	170 F^-	100	—	20	—	370
C	400 Cs, Rb	170 F	100	—	20	—	370
D	400 Na	170 Cl^-	100	—	20	—	370
E	400 Na	170 Cl^-	100	—	20	40, 90	370
F	400 Na	120 Cl^-	—	100	20	80	370
G	400 Na	145 Cl^-	50	50	20	—	370
H	400 Na	145 Cl^-	50	50	20	60, 90	370
I	400 Na	100 Cl^-	50	50	50	—	370
J	400 Na	100 Cl^-	50	50	50	40, 57, 90	370
K	400 Na	170 MS[1]	100	—	20	—	370
L	400 K	170 MS	100	—	20	—	370
M	400 Na	170 MS	100	—	20	90	370
N	400 Na	85 ph[2]	100	—	20	—	370

External solution (mM)

	NaCl	KCl	$CaCl_2$	$SrCl_2$	$MgCl_2$	
α	400	10	100	—	—	Buffered at pH 7·0 by PIPES-Na 5 mM
β	400	10	—	100	—	
Standard ASW	470	10	10	—	50	Buffered at pH 7·8 by Tris-HCl 10 mM

* pH was adjusted at 7·0. [1] Methanesulphonate. [2] Phosphate.

Solutions and Ca buffer system. The compositions of the solutions used in the present experiment are listed in Table 1. *The internal solutions* which were perfused in the lower compartment contained 400 mM-Na, K or Cs as univalent cations, 170 mM-F or 100–170 mM-Cl or 170 mM-methanesulphonate or 85 mM-HPO_4^{2-} as anions. The fluoride ions are known to be the best for stabilizing the membrane and keeping the excitability during the internal perfusion in nerve fibres or ganglion cells (Tasaki, Singer & Takenaka, 1965; Kostyuk *et al.* 1975). A 100 mM-GEDTA (glycoletherdiamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; DOTITE), 100 mM-DPTA-OH (1,3-diaminopropane-2-ol-*N*,*N*′-tetraacetic acid; DOTITE) or 50 mM-GEDTA + 50 mM-DPTA-OH were used as a chelator regulating the amount of free Ca. The pH of the internal solutions were buffered at 7·0 with 20 or 50 mM-PIPES (piperazine-*N*,*N*′-bis[2-ethanesulphonic acid]). By adding $Ca(OH)_2$ up to 90 mM, the free Ca concentration of the internal solution was varied from

zero to millimolar level under the control of the Ca chelator. According to the equation for the binding equilibrium, the free Ca concentration is equal to K'_{Ca}[CaGEDTA]/[free GEDTA], where K'_{Ca} is the dissociation constant of a chelator for Ca (Bjerrum, Schwarzenbach & Sillén, 1957). Since K'_{Ca} of GEDTA at pH 7·0 was calculated as 2×10^{-7} M, it is expected that Ca ions can be fairly well regulated from 2×10^{-8} to 2×10^{-6} M. In order to regulate Ca ions up to millimolar level, it is necessary to use chelating substances with higher K'_{Ca} such as DPTA-OH, whose K'_{Ca} was calculated as 2×10^{-4} M, being capable of regulating free Ca from 2×10^{-5} to 2×10^{-3} M. The absolute stability constants (K_{Ca}s) and the acid dissociation constants ($K_1 \sim K_4$) used for the calculation of K'_{Ca} at pH 7·0 are as follows: For GEDTA, $\log_{10} K_{Ca} = 11$, $pK_1 = 2$, $pK_2 = 2{\cdot}68$, $pK_3 = 8{\cdot}85$, $pK_4 = 9{\cdot}46$ (Holloway & Reilly, 1960) and for DPTA-OH, $\log_{10} K_{Ca} = 6{\cdot}6$, $pK_1 = \sim 2$, $pK_2 = 3{\cdot}36$, $pK_3 = 6{\cdot}85$, $pK_4 = 9{\cdot}70$ (Grimes, Huggard & Wilford, 1963). When the experiment was carried out using DPTA-OH, the zero Ca control experiment should have been done using DPTA-OH without $Ca(OH)_2$. However, it is not certain whether DPTA-OH, with such a high dissociation constant of 2×10^{-4} M for Ca, could reduce Ca ions down below 10^{-8} M as in the case of GEDTA, because there are a few mM of total Ca inside the egg cell. Therefore, the control experiment was carried out by using 100 mM-GEDTA or mixed buffer of 50 mM-GEDTA and 50 mM-DPTA-OH. The pH of the solution is known to affect the dissociation constants of chelating agents such as GEDTA and the neutral Ca salt mixed with Ca buffer solution decreases pH of the solution. Further, the internal pH has some effects on the spike duration in squid giant axons (Watanabe, Terakawa & Nagano, 1977). Thus, in order to keep the Ca concentration as defined by the prescription of the Ca buffer solution, the pH of the solution must be buffered intensively. In the present experiment, 20 or 50 mM-PIPES was used for stabilizing the pH of the internal solutions. The pK_{a2} of PIPES is known as 6·9, indicating that the buffering action should be most powerful around the neutral pH of 7·0. The intracellular Mg concentration has been reported as 5–10 mM (Baker & Grawford, 1972; De Weer, 1976). Thus, Mg may interfere with the stability constant of chelators for Ca. However, both for GEDTA and DPTA-OH, the dissociation constant for Mg was calculated as 5×10^{-3} M at pH 7·0. *The external solution* filling the upper compartment contained 400 mM-NaCl, 10 mM-KCl, and 100 mM-$CaCl_2$ (solution α) or $SrCl_2$ (solution β). The pH was adjusted to 7·0 by 5 mM-PIPES-Na (Table 1). The exchange of the solution in the lower compartment was relatively fast and a 95 % exchange could be assured within 2 min. The temperature of the perfusing solution and of the egg was always kept at 15 °C.

RESULTS

Efficiency of the internal perfusion

Initiation of the internal perfusion. After the egg cell was brought into close contact with the glass wall inside the small funnel (Text-fig. 1), the rupture of the membrane faced to the lower compartment often occurred spontaneously but a high frequency current was also injected through the membrane to facilitate the rupture (see Methods). The moment of the rupture was determined by a sudden increase in the capacity between the two compartments. This increase was expected since the smaller capacity of the lower membrane is connected in series to the capacity of the upper membrane before rupture. The average capacity after rupture was $0{\cdot}99 \pm 0{\cdot}12 \times 10^{-9}$ F ($n = 22$), this value being nearly half of that found in the intact egg (Miyazaki *et al.* 1974). Thus, half of the egg surface appeared to be used for the contact with the glass wall and remaining half, the upper membrane, was used for the voltage-clamp experiment under the intracellularly perfused condition. The contact of an egg with the funnel was photographed in Pl. 1 *A*, *B*, showing that most of the cytoplasm was retained throughout the perfusion.

Immediately after the rupture of the lower membrane, the voltage-clamp experiment was carried out. The holding potential was always set at -90 mV. The holding current was inwardly directed (from the upper to the lower compartment) and the

TABLE 2*A*. The reversal potential of Na Current (internal 400 mM-Na)

Egg no.	Ext. soln. (mM)	Int. soln. (mM)	t_d* (min)	$E_{r.Na}$ (mV)	$I_{h\text{-}90}$ (nA)	$V_{\frac{1}{2}}$ (mV)	$V_{p.max}$ (mV)	$I_{p.max}$ (10 nA)	G_0 (μmho)	d_o† ($\mu \times \mu$)	Remarks
77021801	400 Na	400 Na									
	100 Sr(β)	170 F(A)	21	6	3·0	−53	−35	−1·95	0·49		—
77021802	400 Na	400 Na								80 × 80	
	100 Sr	170 F	11	5	1·9	−51	−35	−3·34	1·03		—
					(K–Na exchange)						
77021501	400 Na	400 Na									
	100 Sr	170 F	30‖	4	0·2	−53	−40	−5·30	1·36	80 × 80	—
					(F solution after Cl solution)						
77022207	400 Na	400 Na									
	100 Sr	170 F	25	6	0·2	−51	−35	−2·28	0·78	—	G_0(F)/G_0(Cl)§ 1·70
77022501	400 Na	400 Na									
	30 Ca‡	170 F	21	6·5	0·7	—	—	—	0·87	70 × 50	1·47
77031206	400 Na	400 Na									
	100 Sr	170 F	40	8·5	0·1	−49	−35	−3·60	1·00	60 × 60	2·33
77031207	400 Na	400 Na									
	100 Sr	170 F	22	6·5	1·5	—	−31·5	−2·20	1·10	70 × 70	2·24
77031208	400 Na	400 Na									
	100 Sr	170 F	31	8·5	0·7	−49	−33	−3·17	1·02	80 × 60	2·00
					(F–MS–F–pH–F exchange)						
77021803	400 Na	400 Na									
	100 Sr	170 F	74	3·5	1·5	—	—	−2·50	0·66	110 × 120	—
Average (F solution)				6·1 ± 1·6		−51	−35	—	—	—	—
					(Na exchange in Cl solution)						
77022203	400 Na	400 Na									
	100 Sr	170 Cl(D)	25·5	9	0·2	−45	−33	−1·72	0·48	70 × 55	—
77022207	400 Na	400 Na									
	100 Sr	170 Cl	12·7	7	0·5	−44	−30	−1·56	0·46	—	—
77022501	400 Na	400 Na									
	30 Ca‡	170 Cl	10	11·5	0·8	−45	−30	−2·30	0·59	—	—
77031206	400 Na	400 Na									
	100 Sr	170 Cl	12	20	1·0	—	—	—	0·43	—	—
77031207	400 Na	400 Na									
	100 Sr	170 Cl	13	7·5	3·5	—	—	—	0·49	—	—
77031208	400 Na	400 Na									
	100 Sr	170 Cl	3	25	1·0	—	—	—	0·51	—	—

* The perfusion time after the rupture except in the case of ‖ where t_d indicates the time after K–Na exchange.
† The diameters of the lower orifice of the funnel.
‡ External solution; 400 mM-NaCl 30 mM-$CaCl_2$, 105 mM-choline Cl.
§ The ratio of the zero current conductance of Na channel in F^- solution to that in preceding Cl^- solution in the same egg.

TABLE 2*B*. The reversal potential of Na current (internal 400 mM-K)

Egg no.	Ext. soln. (mM)	Int. soln. (mM)	t_d* (min)	$E_{r.Na}$ (mV)	$I_{h\text{-}90}$ (nA)	$V_{\frac{1}{2}}$ (mV)	$V_{p.max}$ (mV)	$I_{p.max}$ (10 nA)	G_0 (μmho)	d_o ($\mu \times \mu$)	Remarks
77021701*a*	400 Na	400 K									
	100 Sr	170 F(B)	9	51·5	0·15	−48	−30	−6·50	0·68	—	—
			16	*52*	0·15	—	—	—	0·64	—	—
					(Na–K exchange)						
77021501*b*	400 Na	400 K									
	100 Sr	170 F	65·5	*47*	0·2	−53	−32	−6·00	0·56	—	G_0(K)/G_0(Na)0·41†
					(in MS solution)						
77012102	400 Na	400 K									
	100 Sr	170 MS(K)	1·2	*48*	0·8	−39	−23	−2·85	0·36	—	—
77012103	400 Na	400 K									
	100Sr	170 MS	8	52	0·15	−39	−21	−1·68	0·19	—	—
			30	*48*	—	—	—	—	—	—	—
Average $E_{r.Na}$ (400 mM-K)				48·8 ± 1·9 (n = 4)							

TABLE 2*C*. The reversal potential of Na current (internal 400 mM-Cs)

					(Na–Cs exchange)						
77021802	400 Na	400 Cs									
	100 Sr	170 F(C)	16	68	3·2	—	—	—	0·21	—	G_0(Cs)/G_0(Na)0·20†
			18	75	—	—	—	—	—	—	—
77022207	400 Na	400 Cs									
	100 Sr	170 F	29	78	0·4	—	—	—	0·095	—	0·12
77031208	400 Na	400 Cs									
	100 Sr	170 F	34	83	0·8	−45	−23	−3·40	0·15	—	0·15
Average $E_{r.Na}$ (400 mM-Cs)				78·7 ± 3·3 (n = 3)							

TABLE 2*D*. The reversal potential of Na current (internal 400 mM-Rb)

					(Cs–Rb exchange)						
77031208	400 Na	400 Rb									
	100 Sr	170 F(C)	24	74‡	0·8	—	−22	−2·40	—	—	—

* The perfusion time after Na–K or Na–Cs or Cs–Rb exchange.

† The ratio of the zero conductance to that in the preceding 400 mM-Na solution in the same eggs.

‡ $E_{r.Na}$ in the Cs solution was 83 in the same egg.

TABLE 3*A*. Na and Ca currents with various anions

Egg no.	Ext. soln. (mM)	Int. soln. (mM)	t_d (min)	$E_{r.Na}$ (mV)	$I_{h\text{-}90}$ (nA)	Na current $V_{\frac{1}{2}}$ (mV)	Na current $V_{p.max}$ (mV)	Na current $I_{p.max}$ (10 nA)	Na current G_0 (μmho)	Sr or Ca current $V_{\frac{1}{2}}$ (mV)	Sr or Ca current $V_{p.max}$ (mV)	Sr or Ca current $I_{p.max}$ (nA)
					(MS solution)							
77011410	400 Na	400 Na										
	100 Sr(β)	170 MS	8	26	0·15	−39	−21	1·42	0·35	−2	15	3·8
					(phosphate solution)							
77021804	400 Na	400 Na										
	100 Sr	85 ph	6	26	0·15	−41	−30	1·58	0·33	−3	10	3·8
					(Cl solution)							
77031201	400 Na	400 Na										
	100 Sr	170 Cl	7	25	0·2	—	—	—	0·40	−7	7·5	5·4
77031202	400 Na	400 Na										
	100 Sr	170 Cl	8·5	26	0·2	−36	−21	2·12	0·52	—	15	2·0
77031209	400 Na	400 Na										
	100 Sr	170 Cl	6·2	25	0	—	—	—	0·66	−1	15	3·8

TABLE 3*B*. Na and Ca current in the intact eggs

					(Na and Sr currents in Sr ASW)							
73121701	400 Na											
	100 Sr(β)	—	—	—	0·1	−41	−23	0·76	—	−7	6	12·5
75032501	100 Na											
	100 Sr	—	—	27	0	−42	−22	0·44	—	−4	13	4·6
77031001	400 Na											
	100 Sr	—	—	—	0·2	−35	−18	4·00	—	0	15	5·4
Average						−39·3	−21			−3·7	11·3	
					(Na and Ca currents in Ca ASW)							
72081501	400 Na											
	100 Ca(α)	—	—	—	—	−37	−15	0·41	—	+7	23	1·7
73121701	400 Na											
	100 Ca	—	—	—	0·1	−33	−20	0·62	—	+4	19	7·2
75032501	100 Na											
	100 Ca	—	—	28	0·4	−35	−19	0·45	—	+8	24	2·6
Average						−35	−18			+6·6	22	

amount was less than 1 nA in most of the experiments with various compositions of internal and external solutions. In the successful cases, the holding current was less than 0·2 nA, this value being similar to that observed in the intact egg in the comparable external medium (Tables 2, 3*B*). Considering that the upper membrane was half of the whole, it was suggested that the insulation between the egg and the glass wall was virtually perfect.

The Na reversal potential as an indicator of the solution exchange. As previously reported, the egg cell membrane of the tunicate shows a transient inward Na current in response to a depolarizing pulse (Okamoto *et al.* 1976*a*). The reversal potential for the Na current is around +60 mV in standard medium containing 460 mM-Na ions. This suggests that the concentration of Na ions inside the egg cell is roughly 50 mM or one tenth of the outside concentration, if the Na channels are permeable only to Na ions. It is expected, therefore, that if internal perfusion is started with a perfusate of such a high Na content as 400 mM, the progress of the intracellular solution exchange can be known as a negative shift in the Na reversal potential.

Text-fig. 2*A* illustrates a family of membrane currents recorded from an egg in external solution β after 40 min internal perfusion with a solution containing 400 mM-Na^+ and 170 mM-F^- (solution A). These currents, in response to step changes of the membrane potential in the positive direction applied to the holding potential of −90 mV, revealed transient inward currents and small steady currents which are very similar to Na current and K current observed in the intact egg cell (Okamoto *et al.* 1976*a*).

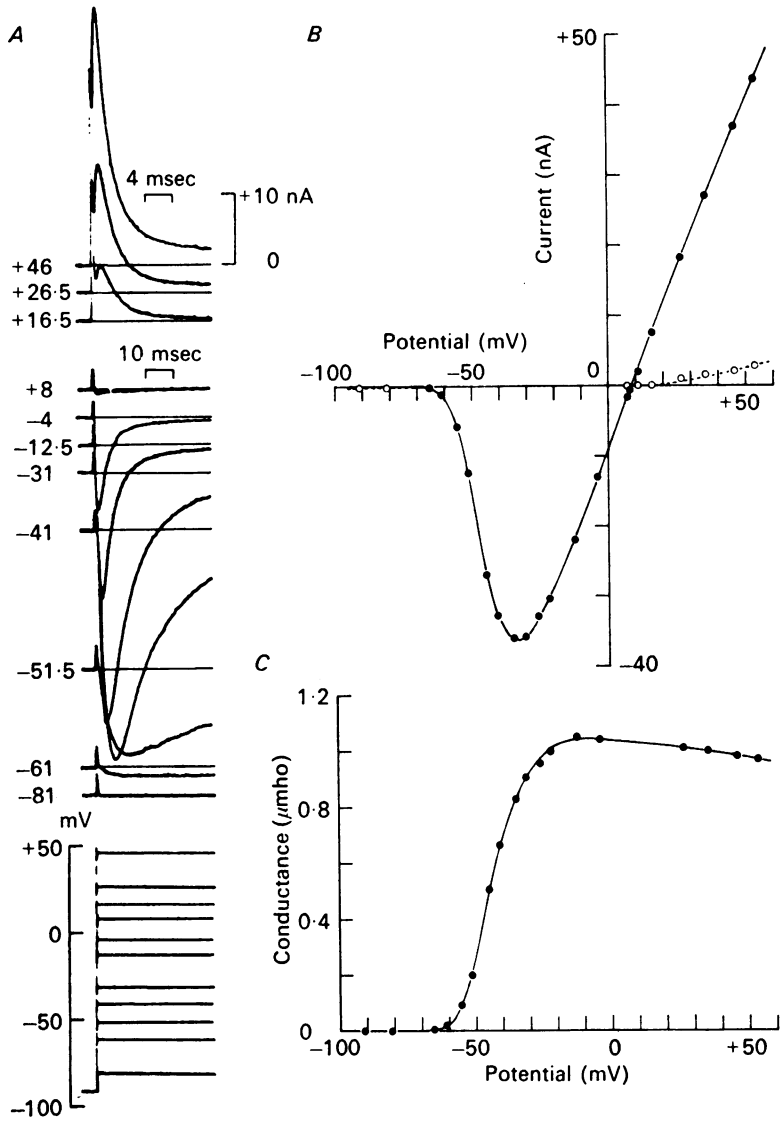
The transient inward current appeared when the membrane was depolarized above −65 mV and the peak of the current attained its maximum at −35 mV, as shown in Text-fig. 2*B*. The reversal potential for the transient inward current was +8·5 mV (Text-fig. 2*A*, *B*). Since Na concentration was the same in both internal and external solutions, the result suggests that the exchange efficiency was 71 %.

The steady-state current, which probably corresponded to the K current, was also included in Text-fig. 2*B* with open circles. As expected from the scarcity of K ions in the internal perfusate, the steady current at potentials less positive than +30 mV was almost negligible and within 3 nA even at a level of +50 mV.

Unlike the Na current, the slow 'Ca channel' current due to Ca or Sr ions, which is usually observed in the intact egg at potentials above −10 mV with 100 mM-Ca or Sr medium (Table 3*B*), was abolished completely by using F^- ions in the internal perfusate. Therefore, it appears that the observed transient inward current was not contaminated with Ca channel current and its polarity change directly indicates a reversal of the Na current.

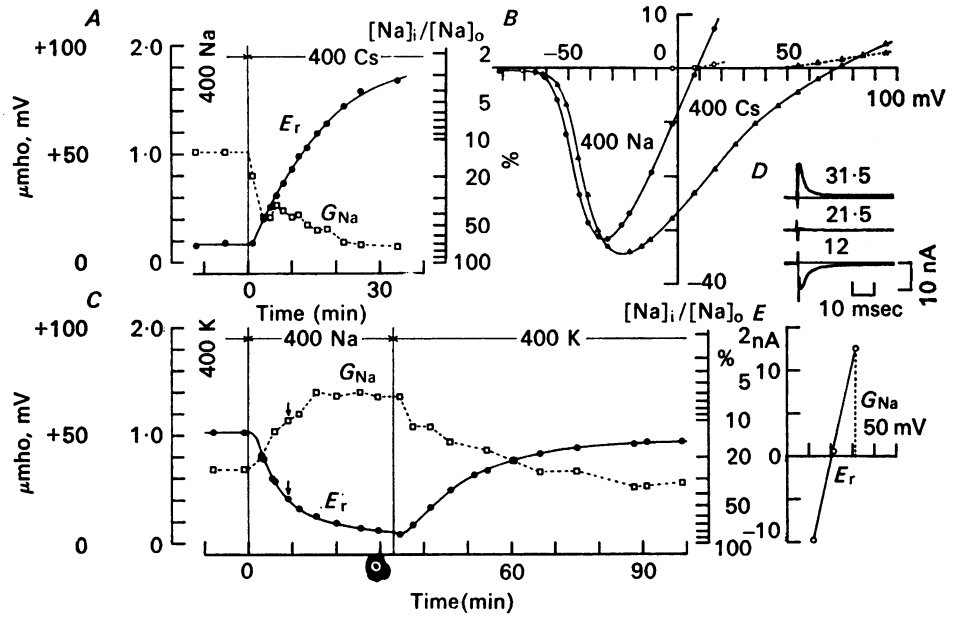
The time course of the internal perfusion. Using the reversal potential for the Na current (E_r) as an indicator of the intracellular concentration of Na ions, the time course of solution exchange was investigated. If the Na channel of the tunicate egg is only permeable to Na ions, the reversal potential (E_r) can be predicted by the Nernst equation: $E_r = (RT/F)\ln([Na]_o/[Na]_i)$, where $[Na]_i$ and $[Na]_o$ are the internal and the external Na concentrations respectively, and R, T and F have their usual meaning.

The E_r of the Na current was determined by applying step changes of the potential around the zero current level, measuring the peaks of the currents (Text-fig. 3*D*)



Text-fig. 2. Transient membrane currents and their potential dependence in the internally perfused egg. *A* illustrates various current traces with potential traces under the voltage-clamp condition. The external solution, 400 mM-Na and 100 mM-Sr (solution β) and the internal perfusate, 400 mM-Na and 170 mM-F^- (solution A). The holding potential, −90 mV. The potential of the test pulse is indicated with the number (mV) by each current trace. The horizontal line for each current trace indicates zero current level. The upward direction is inward and the downward is outward. The time scale for currents is also applied to potential records. *B*, the current–voltage (*I–V*) relations for the peak of the transient current (●) and for the steady-state current (○) from the same data as illustrated in *A*. *C*, the chord conductance for the peak of the transient current is plotted against the membrane potential. The reversal potential is +8·5 mV. Perfusion time after rupture, 40 min. Temperature, 15 °C. Egg no. 77031206.

and plotting the $I-V$ relations (Text-fig. 3*E*), the zero current intercept giving E_r. At the same time the Na conductance at the zero-current level (G_{Na}) was calculated from the slope of the $I-V$ relationship, shown in Text-fig. 3*E*. Both E_r and G_{Na} are plotted against time in Text-fig. 3*C*.



Text-fig. 3. Changes of reversal potential (E_r) and zero-current conductance G_{Na} of Na current during exchange of intracellular cation content. *A*, changes in Na reversal potential (E_r) and zero-current Na conductance (G_{Na}) when internal 400 mM-Na (solution A) was replaced with 400 mM-Cs (solution C). The external solution was unchanged, containing 400 mM-Na (solution β). Ordinate (left), indicating the membrane potential (mV) and the conductance (μmho). Ordinate (right), indicating the ratio (%) of the internal to the external concentration of Na ions expected from E_r, according to the Nernst equation for Na ions. Abscissa, the perfusion time (min) after Na/Cs exchange. The time from rupture in Na perfusate to initiation of Cs perfusion was 35 min. Egg no. 77031208. *B*, $I-V$ relations for the peak of the Na current at saturated states in Na perfusion (●) and in Cs perfusion (▲) from the same data in *A*. The steady-state currents for Na perfusion (○) and Cs perfusion (△) are also included. Each crossing between Na current and steady state gives E_r. *C*, changes in E_r and G_{Na} when internal perfusate was changed in the sequence of 400 K, 400 Na and 400 K perfusates (solution B, A and B). The external solution was unchanged (solution β). Ordinates and abscissa are the same as in *A*. The perfusion time of the initial K phase was 20 min. Egg no. 77021501. *D* illustrates samples of the current traces around reversal which were used as measure of E_r and G_{Na} in *C* (arrows). The peak of the current is plotted in *E* against the membrane potential, to illustrate the measurements of G_{Na} and E_r.

In the experiment presented in Text-fig. 3*C*, the internal perfusion was started with the solution B containing 400 mM-K and later the perfusate was replaced with the solution A containing 400 mM-Na and returned to the initial perfusate 400 mM-K solution again. For 20 min after rupture, E_r for 400 mM-K perfusate stayed around +51 mV, the level being similar to the intact egg. The replacement with 400 mM-Na perfusate at the time indicated as 0 min caused a negative shift in E_r, the decay

of which with time was linear down to +30 mV and thereafter roughly exponential to a saturation level of +4 mV which was attained 30 min after exchanging the perfusate. The average Na reversal potential was $+6{\cdot}1 \pm 1{\cdot}6$ mV ($n = 9$) with the 400 mM-Na perfusion of 10–40 min (average 25 min) either after perfusate exchange or after rupture (Table 2*A*). The result indicates that the intracellular Na concentration reached 80 % (range 71–87 %) of the Na level of the internal perfusate within 25 min. When the perfusate was returned to the initial 400 mM-K solution, the original E_r around +50 mV was again observed within 60 min. The G_{Na}, which had once increased in 400 mM-Na perfusate, also returned to the original level. The change in G_{Na} will be described later.

Some characteristics of Na channels examined by the intracellular perfusion technique

Na current and its current–voltage relationship with high Na perfusate. As shown in Text-fig. 2*A* and *B*, the egg cell perfused with solution A (the external solution β) revealed inward Na currents with depolarizations above −65 mV as in the intact egg, while the reversal potential being shifted in the negative direction. The peak of the Na current became maximum around −35 mV ($V_{p.max.}$), which ranged between 30 and 50 nA (Table 2*A*). The potential level at which the peak of the current attains its half-maximum ($V_{\frac{1}{2}}$) was −51 mV (Table 2*A*). The peak time of the inward Na current was 20 msec at −50 mV and became shorter with depolarization being 3·5 msec at −35 mV (Text-fig. 2*B*). Above +20 mV the peak time was saturated being 1·5 msec. Thus, the potential dependences of the current magnitude and the time course were similar to those in the intact egg in a comparable external solution, such as a medium containing 100 mM-$SrCl_2$ and 400 mM-NaCl. However $V_{\frac{1}{2}}$ and $V_{p.max.}$ were both shifted in the negative direction by about 10 mV in the perfused egg (Tables 2*A*, 3*B*). The cause of this shift in $V_{p.max.}$ appears mainly due to the negatively shifted reversal potential due to the high internal Na concentration, while the shift in $V_{\frac{1}{2}}$ and possibly that in $V_{p.max.}$ were found due to fluoride ions in the internal solution, as will be described later. Actually similar shifts of $V_{\frac{1}{2}}$ and $V_{p.max.}$ were observed with the internal perfusate containing 400 mM-K and 170 mM-F^- as well (Table 2*B*).

With 400 mM-Na perfusate, the conductance around the reversal level was increased (Text-fig. 3*C*) and almost constant with respect to the membrane potential, the I–V relation being linear above −20 mV (Text-fig. 2*B*, 3*B*). In contrast to this observation a moderate non-linearity has been found in the intact cell (Okamoto *et al.* 1976*a*). The $V_{\frac{1}{2}}$ with high Na perfusate could therefore be estimated also from the chord conductance–potential curve. The chord conductance at the peak Na current (I_p) was calculated from the formula $I_{Na.p}/(V - E_r)$ and plotted against the membrane potential in Text-fig. 2*C*. The conductance increased abruptly in the potential range from −60 to −20 mV, forming an S-shaped curve. The saturated chord conductance at −20 mV was 1·02 μmho and the half-maximum conductance was obtained at −45 mV. The maximum slope of the conductance change, which occurred near $V_{\frac{1}{2}}$, was $0{\cdot}5 \times 10^{-4}$ mho/V. The general shape of the conductance curve is similar to that obtained for Na current in the intact egg (Okamoto *et al.* 1976*a*).

However, there was a voltage shift of the curve in the negative direction as in the *I–V* relation along the potential axis.

Selective permeability of the Na channels to Na, K and Cs. The average reversal potential for Na current with internal 400 mM-K perfusate (solution B or L) was +49 mV ($n = 4$, Table 2*B*), this value being about 10 mV less positive than that in the intact egg in standard medium containing 460 mM-Na (Okamoto *et al.* 1976*a*). The fact that a finite reversal potential was obtained with internal K perfusate without Na (Text-fig. 3*C*), suggests that Na channels of the tunicate egg also have a permeability to K ions, rather than that of the removal of the intracellular Na is incomplete (Hille, 1970). As reported previously, the Na channel of the tunicate egg is permeable to K ions, the degree being one tenth compared to the permeability to Na ions (Okamoto *et al.* 1976*a*). The more positive reversal potential (about +60 mV) observed in the intact eggs in standard ASW may be partly due to a higher external Na concentration (460 mM in comparison with 400 mM) and partly due to a lower intracellular K concentration (200 mM in comparison with 400 mM; Miyazaki *et al.* 1974) than in the perfused egg cell.

The internal Na perfusate was changed to Cs perfusate (solution C) and the results are shown in Text-fig. 3*A* and Table 2*C*. Since Na channels are known to be much less permeable to Cs ions (Hille, 1970), the reversal potential shifted to more than +80 mV within 30 min after solution exchange (Table 2*C*). Since the E_r of +80 mV corresponds to the concentration ratio of 0·04, the permeability ratio (P_{Cs}/P_{Na}) must be 0·04 at most when the solution exchange is perfect. Alternatively if one assumes Cs as an impermeant ion, the exchange efficiency will be 96 % with 30 min perfusion. The efficiency will be more than 96 % when Cs ions are slightly permeant. The exchange efficiency of 96 % was much larger than that of 80 % obtained by perfusion with internal high Na content, as described above. The wash-out process may be more efficient than the increasing process of Na concentration.

Using the Goldman–Hodgkin–Katz equation, the permeability sequence was Na > K > Rb > Cs, the ratio being 1:0·14:0·05:0·04 (Tables 2*B*, *C*, *D*). This sequence and the ratio are similar to those found in the Na channels of other excitable membrane (Hille, 1970; Table III in Ebert & Goldman, 1976) and to those estimated in the intact tunicate egg by changing external cationic compositions (Okamoto *et al.* 1976*a*).

As shown in Text-figs. 3*A* and *C*, the Na channel conductance at zero current level (G_{Na}) became two to three times greater when internal K perfusate was replaced with Na perfusate, and was five to six times greater in Na perfusate than in Cs perfusate (Tables 2*B*, *C*). Therefore, the conductance ratios were approximately proportional to the square root of the permeability ratios only when the internal perfusate was changed. This relationship is just as expected from the dependence of the conductance upon the ion concentration and the permeability coefficient on one side of the membrane (S. Ciani, S. Krasne, S. Miyazaki & S. Hagiwara, personal communication).

Text-fig. 3*B* illustrates the current–voltage relations for the peak of the Na current, before and after changing 400 mM-Na perfusate to 400 mM-Cs perfusate. In the Na perfusate almost no rectification was found around the reversal potential as described before, while with internal Cs ions, to which Na channels are scarcely

permeable, a considerable inward rectification was noticed, being consistent with a decreased conductance for internal ions. The change in the reversal potential during the Na–Cs exchange indicated a monotonous increase and decrease in the intracellular Cs and Na concentrations, respectively (Text-fig. 3*A*). G_{Na}, however, always went through a minimum when $\frac{1}{3}$ to $\frac{1}{2}$ of the Na ions were considered to be replaced by Cs ions. This minimum may be due to ionic interactions within the Na channels as having been demonstrated in several other ionic channels (Takeuchi & Takeuchi, 1971; Hagiwara & Takahashi, 1974; Hille, 1975).

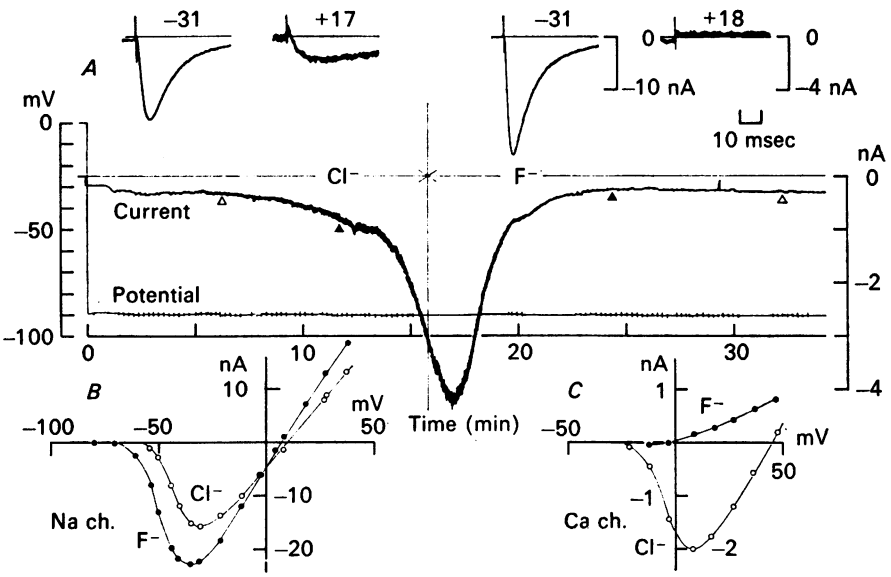
Supposing that the solution exchange in the intracellular compartment is a simple diffusional process and that the reversal potential is well described by the Nernst equation for Na during the initial phase of Na-Cs exchange, then the slope of the reversal potential change (dE_r/dt) would be constant and equal to $(RT/F).1/\tau_d$, where τ_d is the time constant of the exchange and (RT/F) is 25 mV at 15 °C. Actually the initial time course of the exchange was relatively linear, as shown in Text-fig. 3*A*, with a time constant of about 10 min. The time constant varied slightly according to the size of the lower orifice of the funnel.

Comparison of the effects of the internal Cl^- ions upon Na and Ca channels with those of the internal F^- ions

Abolition of Ca current by internal F^- perfusate. As demonstrated previously, F^- ions stabilized the egg membrane and kept its excitability for a long period of time during the internal perfusion, while Ca current, which has been found in the intact egg, never appeared in any internal F^- perfusates (solutions A–C). Since it was our aim to analyse both Na and Ca currents as a function of the internal free Ca concentration, we attempted to use other anions which would preserve Na and Ca currents as in the intact egg. Among various anions (such as phosphate, methanesulphonate, sulphonate, and chloride), Cl^- ions were found useful for this purpose because not only are both currents observed when the perfusate contains Cl ions, but also there is no interaction with free Ca ions up to a concentration of 1 mM. With internal Cl^- perfusate, however, the preparation remained in good condition only for 20–30 min.

Text-fig. 4 demonstrates that significant differences exist in the behaviours of various membrane currents in the presence of Cl^- ions *vs.* F^- ions in the internal perfusates (solutions A and D). In Text-fig. 4*A*, the lower trace indicates the membrane potential, and the upper one the holding current. Immediately after rupture, the membrane potential was held at −90 mV. The initial perfusate was Cl^- solution and later replaced by F^- perfusate as indicated in Text-fig. 4*A*. During the initial 10 min, the holding current was as low as 0·3 nA even in the Cl^- perfusate, but thereafter increased gradually and at some point around 20–30 min, increased abruptly, as shown in Text-fig. 4*A*. Generally, whenever the external solution was α or β (β was the case of Text-fig. 4), the holding current in the Cl^- perfusate was usually little and relatively stable within the initial 10–15 min. When Cl^- perfusion was continued a few minutes after such a marked increase in the holding current, the membrane became so leaky that experiments were terminated. However, when the internal Cl^- perfusate was changed to F^- perfusate just before the destruction of the membrane, the holding current, which had once increased, decreased again and finally became stable at a low level of less than 0·2 nA (Text-fig. 4*A*).

During the phase of the relatively stable and low holding current in Cl^- perfusate, step changes in the potential revealed both Na and Ca current. As previously reported (Okamoto *et al.* 1976*a*, *b*), Ca current in the intact egg is easily isolated from Na current by applying a preceding depolarization which inactivates Na current, because there is a wide difference of the inactivation levels of 40 mV between Na and Ca currents. In the egg cell internally perfused with the Cl^- solution, Ca current was similarly isolated from Na current with the same procedure. Essentially the same results were obtained when Ca was substituted by Sr. Sr was a good substitute in the Cl^--perfused egg as in the intact egg. The 'Ca channel' current carried by Sr (Sr current) is illustrated in Text-fig. 4 (left inset of Text-fig. 4*A*). After changing the perfusate into F^- solution, the Sr current was completely abolished, as shown in the right inset of Text-fig. 4*A*.



Text-fig. 4. The comparison of the holding current, Na current and Sr current between internal Cl^- and F^- perfusion. All through the experiment the external solution was β. *A* illustrates the holding current at -90 mV and the membrane potential on the multichannel DC-recorder in an egg cell perfused initially with Cl^- solution (D) and later with F^- solution (A). Insets are sample records of Na current at -31 mV and Sr current at $+17$ mV isolated from Na current by conditioning depolarizing pulse of 400 msec to -34 mV in Cl (left side) and F (right side) perfusates. The times when the samples were obtained were indicated by triangles on the DC current records. △ for Na currents and ▲ for Sr currents. Egg no. 77022207. *B* and *C* illustrate *I–V* relations of Na and Sr currents respectively with internal Cl (open circles) and F (filled circles) perfusates. Sr currents were also isolated by conditioning depolarization to -34 mV. The egg no. was the same as in *A*.

The current–voltage relations at the peaks of Na and Sr currents are presented fo internal F^- (filled circles) and Cl^- (open circles) perfusates in Text-fig. 4*B* (Na current) and 4*C* (Sr current) from the same experiment as shown in Text-fig. 4*A*. The maximum inward Na current in F^- perfusate was usually 1·5–2 times larger than that in Cl^- perfusate (1·4 times in case of Text-fig. 4*B*, Table 2*A*). The maximum

current in Cl^- perfusate with external solution β was 10–20 nA. The reversal potential of the Na current in Cl^- perfusate was shifted in the negative direction just as in F^- perfusate when the perfusate contained 400 mM-Na and the time after rupture was more than 10 min (Table 2*A*). In the case of Text-fig. 4*B*, the reversal potential in F^- perfusate was +6 mV and slightly more negative than that of +9 mV in Cl^- perfusate. This is simply due to the longer perfusion time for the recordings in F^- perfusate.

As shown in Text-fig. 4*B*, the $V_{\frac{1}{2}}$ and the $V_{p.\,max.}$ of the Na current in F^- perfusate was clearly shifted in the negative direction by 5–10 mV in comparison with those in Cl^- perfusate. Those values in Cl^- perfusate were similar to those found in the intact eggs (Tables 2*A*, 3*A*, *B*). This corresponds to the fact that the $I-V$ relation of the Na current in the F^--perfused egg was shifted to the negative along the voltage axis compared to that in the intact egg as described before.

The $V_{\frac{1}{2}}$ and the $V_{p.\,max.}$ of the Ca or Sr current in Cl^- perfusate were also similar to those in the intact egg (Tables 3*A*, *B*). The maximum currents for the 'Ca channel' in Cl^- perfusate were usually 2–5 nA with the external solution β (Sr current) and 2–3 nA with external solution α (Ca current), although, in case of Text-fig. 4*C*, the Sr current was less than the average (Tables 3*A*, 4, 5*A*). In F^- perfusate the 'Ca channel' current was completely abolished and only the outward current was observed, as shown in Text-fig. 4*C* (F^-).

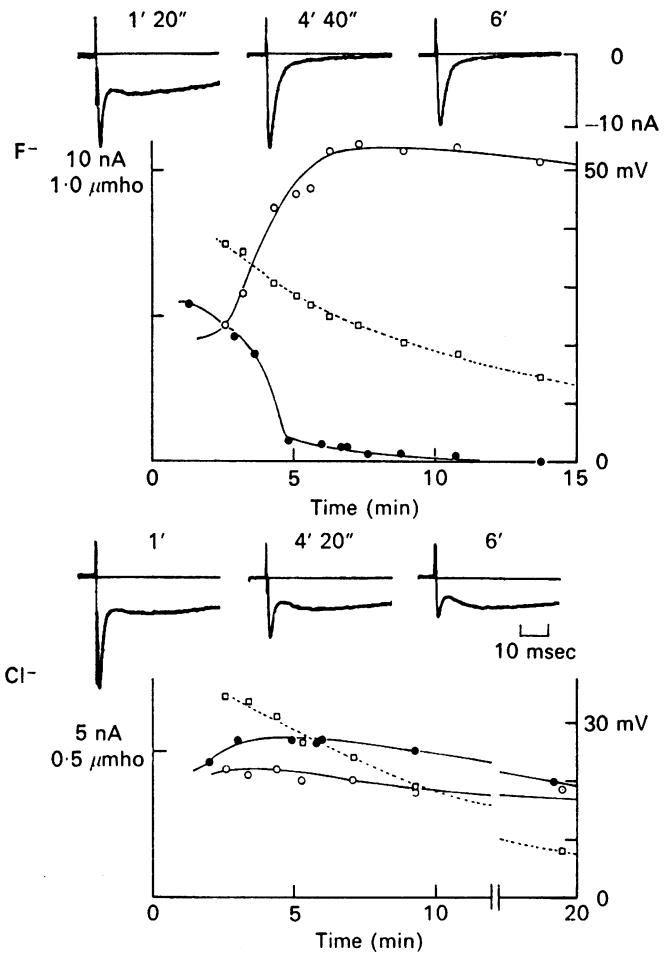
Methanesulphonate or phosphate internal perfusate (solutions K to N) was found to be equally useful for keeping both Na and Ca currents as Cl solution (Tables 3*A*, 5*B*), although phosphate interacts with free Ca of concentration above 10^{-5} M.

All of the above results suggest that the Cl^- or non-F^- perfusate is more suitable for studying both Na and Ca currents than the F^- perfusate. However, because the membrane usually became leaky 10–15 min after introducing the Cl^- or non-perfusate, the transient phase of exchange was used for the following experiments with the Cl^- perfusate.

Na and Ca currents during the transient phase of exchange. Text-fig. 5 illustrates typical examples of the changes occurring in Na and Sr currents during the initial phases of F^- and Cl^- perfusion. The reversal potential of Na current (open squares), the zero-current Na conductance (open circles), and the maximum Sr current (filled circles) were plotted against the time after rupture. The reason why G_{Na} instead of the maximum Na current was taken as measure of the conductivity for Na channels is as follows: (1) Since the high Na content in the perfusate shifts the Na reversal potential in the negative direction, the amplitude of the inward Na current is markedly reduced around $V_{p.\,max.}$ with the progress of the exchange. (2) The Na chord conductance defined by the formula, $I_p/(V-E_r)$, especially at the zero-current level (G_{Na}) is known to be relatively insensitive to the changes in the internal Na concentration, as described before.

The insets in the upper (F^-) and lower (Cl^-) figures in Text-fig. 5 are sample current records in response to step changes in potential to +20 mV. Since no conditioning depolarization was applied, the recorded trace shows both initial fast Na and later slow Sr currents. Within 1–2 min after initiation of the perfusion, the E was usually above +40 mV suggesting that in this initial period the intracellular medium is still almost the same (less than 20 % exchange) as in the intact egg. In this initial period,

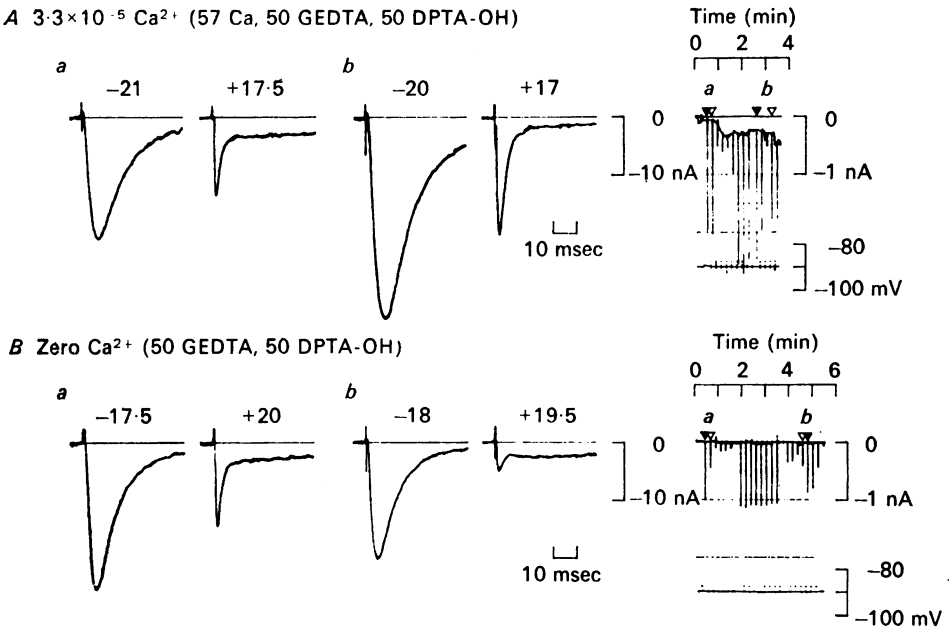
each record for F^- or Cl^- perfusate shows a similar shape consisting of initial Na and later Sr currents. With the progress of exchange, as indicated by E_r change, the Sr current was abolished in F^- perfusate, while the G_{Na} doubled. However, in Cl^- perfusate



Text-fig. 5. The changes of Na reversal potential ($E_{r,Na}$, open squares), peak amplitude of Ca current (I_{Ca}, filled circles) at +17 mV, and Na conductance at zero current level (G_{Na}, open circles) during the transient phases of F^- (upper Figure) and Cl^- (lower Figure) perfusion. External solution, β. Internal solutions are A(F) and D(Cl). Ordinate: nA for I_{Ca} and μmho for G_{Na}. Abscissa: the perfusion time after the rupture or initiation of the perfusion. Insets are sample current records at +17 mV. Since no conditioning depolarization preceded, both initial fast Na and later slow Ca currents were observed. The number on each trace indicates the perfusion time when the record was obtained. Egg no. 77031205 for upper Figure (F) and 77031201 for lower Figure (Cl).

both G_{Na} and the maximum Sr current stayed relatively constant. Fifty to one-hundred mM-F^- ions seems to be necessary to abolish Ca current because E_r in this experiment indicates a 30–50% exchange. The time course of E_r change for F^- perfusate was almost identical with that for Cl^- perfusate, suggesting that the intracellular diffusion is similar between two perfusates.

Both G_{Na} and the maximum Sr current in the Cl^- perfusate consistently showed a tendency to decrease gradually in several minutes after rupture. The decrease, however, was not significant within about 10 min after initiation of the perfusion or until E_r reached +20 mV. Thus, in the following experiments with internal 400 mM-Na, Cl^- perfusate, the effect of the intracellular free Ca on the Na and Ca channels was studied over the range in which the Na reversal potential stayed between +40 and +20 mV.



Text-fig. 6. Effects of internal Ca ion upon Na and Ca currents. *A* illustrates current traces immediately after rupture (*a*) and 2·7–3·2 min later (*b*) in the presence of $3·3 \times 10^{-5}$ M-Ca ions in the internal perfusate (solution J). The external solution contained 400 mM-Na and 100 mM-Ca (solution α). The attached number above each current trace indicates its membrane potential. The inset shows d.c. records of current (upper) and potential (lower). The zero time on the time scale indicates the time of rupture and the times when illustrated current traces were obtained are also indicated (▼, −20 mV trace; ▽, +17 mV trace). Egg no. 77050804. *B* shows control experiment on another egg in the absence of Ca ions in the internal perfusate (solution I). The external solution was the same as in *A*. All symbols, numbers and scales also have the same meaning as in *A*. Egg no. 77050801.

Differential effect of the intracellular free Ca ions upon Na and Ca channels

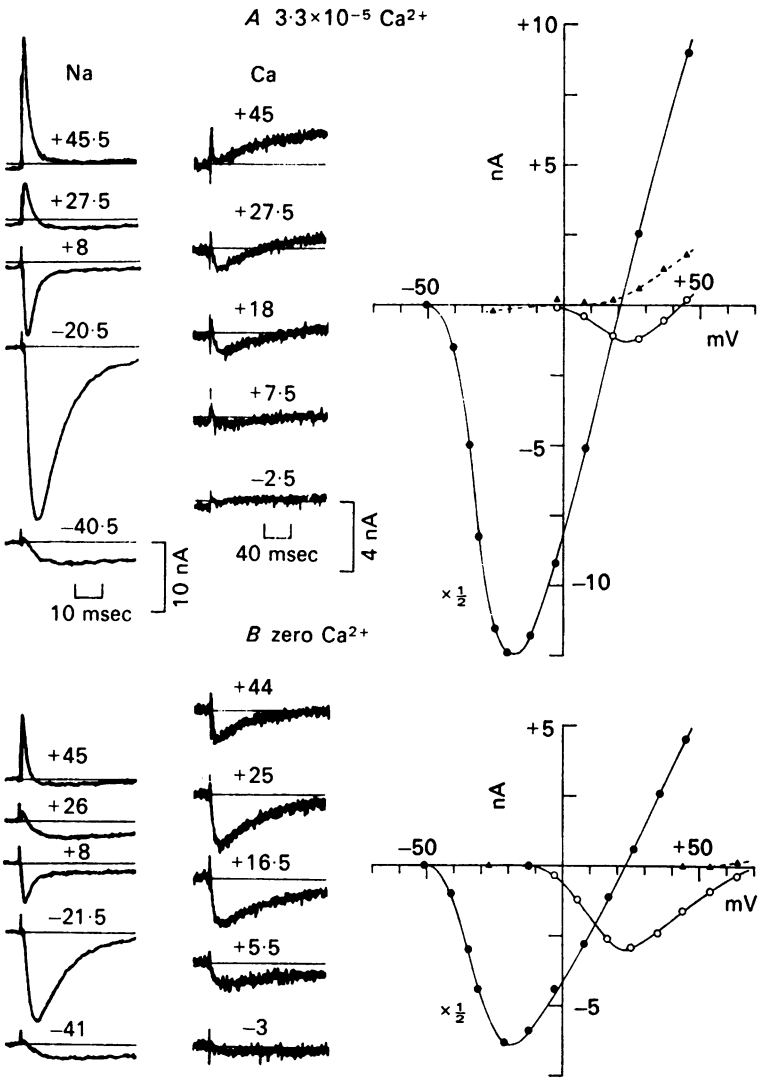
Reciprocal effect of the internal Ca ions upon Na and Ca currents. It is known that the intracellular Ca ions have an inhibitory effect upon the Ca current through Ca channels (Hagiwara & Nakajima, 1966). In the present experiment, the effect was tested by perfusing the Ca ion-buffered solution intracellularly. Text-fig. 6*A* illustrates an experiment in which an egg was perfused with solution *J* containing a relatively high concentration of free Ca ions of $3·3 \times 10^{-5}$ M. The external solution α was kept constant throughout the experiment. The time course of the perfusion is shown in the inset; the perfusion being started at zero-time and the records *a* and *b*

being obtained at the times indicated. The filled triangles indicate the times when the currents at -20 mV were induced (to observe the maximum Na current) and the open triangles indicate the times when the currents were induced at $+17$ mV (to observe the maximum Ca current). Within the initial 1 min when the record *a* was obtained, the observed Na and Ca currents were identical to those in the control perfusate with zero Ca (Text-fig. 6*B*, *a*). A few minutes after initiation of the perfusion of $3{\cdot}3 \times 10^{-5}$ M-Ca ions, the holding current suddenly increased by a factor of three to five (inset in Text-fig. 6*A*), whereas the Na current nearly doubled and the Ca current was reduced to one half. This effect was dramatic and consistent. The time courses of such increased Na and decreased Ca currents were not significantly different from those observed during an initial few minutes. Thus, it seems likely that the chord conductance, but not the gating mechanisms, were affected. The holding current, having once increased, stayed at a relatively high level and often started to increase further after 5–10 min. The later increase was likely to be due to greater fragility of the membrane in high free Ca perfusate. As a control for the Ca experiment, the solution I, which contained the same amount of the chelating agents but zero Ca, was perfused, for which the results are shown in Text-fig. 6*B*. In this case, the holding current and the Na and Ca currents which were observed within the initial 1 min (Text-fig. 6*B*, *a*) were not significantly different from those obtained several minutes later (Text-fig. 6*B*, *b*), or if any there was a tendency towards a decrease of both Na and Ca currents. The holding current stayed at the same low level without showing any abrupt increase (inset in Text-fig. 6*B*). Therefore, it is concluded that, at a concentration more than 10^{-5} M, the intracellular free Ca ions have a facilitatory effect upon the Na current and upon the holding current while they show an inhibitory effect upon the Ca current. An increase in the holding current may be due to a Ca-mediated increase in the K conductance at the resting level, as has been suggested in several excitable membranes (Krnjević & Lisiewicz, 1972; Meech, 1974; Yamagishi, 1977).

The current–voltage relations of Na and Ca currents with internal high free Ca perfusate. To observe the effect of intracellular free Ca upon the potential dependences of Na and Ca currents, these currents were recorded as a function of the membrane potential for two different Ca concentrations in the internal perfusates (Text-fig. 7). To eliminate the effect of different Na concentrations inside the egg upon the I–V relation, the comparison was made between the egg cells which showed similar reversal potentials of Na current. In the case of Text-fig. 7, E_rs were $+21$ and $+23$ mV for $3{\cdot}3 \times 10^{-5}$ M free Ca (*A*) and zero free Ca perfusates (*B*) respectively.

As shown by the current records in Text-fig. 7, the Na currents in the $3{\cdot}3 \times 10^{-5}$ M free Ca perfusate were exactly the same in their time courses as those in zero free Ca perfusate at any levels of the membrane potential, whereas their magnitude was doubled at any range of the potential. The comparison between the I–V relations for the peak of the Na current (filled circles) reveals that the potential dependence of the amplitude of the Na current also remained unchanged with the increase in intracellular free Ca.

The Ca currents isolated from the Na currents with a prepulse to -27 mV are also illustrated in Text-fig. 7. When the Ca current records in Text-fig. 7*A* and *B* were compared, it was shown that the time courses of the Ca currents were identical with

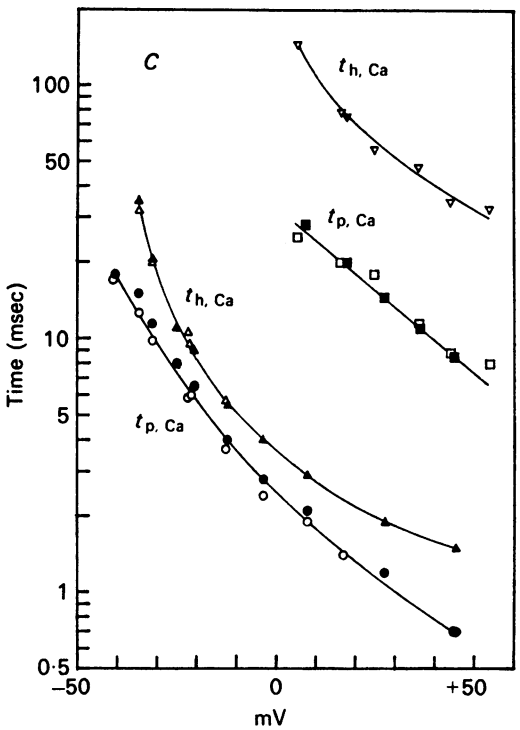


Text-fig. 7. Effects of internal Ca ion upon the potential dependence of Na and Ca currents. *A* illustrates Na current traces (left) and Ca current traces (right) at the 6 min perfusion with $3{\cdot}3 \times 10^{-5}$ M-Ca ions (solution J). The external solution contained 400 mM-Na and 100 mM-Ca (solution α). The number attached to each current trace is its membrane potential (mV). For Ca current, a conditioning pulse of 400 msec to −26 mV was applied to inactivate Na current. Their *I–V* relations for the peak of the current (●, Na current; ○, Ca current) and for the steady-state current (▲) are also shown in *A*. Egg no. the same as Fig. 6*A*. *B* shows a control experiment on other egg in the absence of Ca ions in the internal perfusate (solution I). The perfusion time after rupture was 6 min. The external solution was the same as in *A*. All symbols, numbers and scales have the same meaning as in *A*. Egg no. 77050806. *C* illustrates the peak and the half decay times of Na and Ca currents with zero (open symbols) and high free Ca (filled symbols) perfusates. The analysed records are the same as illustrated in *A* and *B*.

each other at the same membrane potential either with high Ca or zero perfusate. The *I–V* relations for the peak of the Ca current (open circles) appear to be essentially the same except that the magnitude of the Ca current in $3{\cdot}3 \times 10^{-5}$ M free Ca perfusate became half of that in zero Ca perfusate.

Both peak (t_p) and half decay (t_h) times of Na and Ca currents are plotted against the membrane potentials for the cases of zero (open symbols) and high internal Ca (filled symbols) in Text-fig. 7*C*. The result further confirms that the time courses of the currents are quantitatively identical between zero and high free Ca perfusates.

In a high free Ca perfusate, an increase in the outward current at the steady state was frequently observed as shown in Text-fig. 7*A* (filled triangles) compared with that in the zero Ca perfusate shown in Text-fig. 7*B*. However, this effect was variable so that we could not decide whether the real increase in the K conductance or enhanced leakage current was responsible.



Text-fig. 7*C*. For legend see facing page.

In summary, the potential dependence of neither time course nor amplitude of Na and Ca currents was significantly changed by the intracellular free Ca, but the relative magnitude of the currents was affected. The collected data from *I–V* relations of Na and Ca currents in zero and high free Ca perfusates are listed in Table 4.

The Na conductance and the maximum Ca current as a function of intracellular free Ca. As described in the preceding section, only relative magnitude of the current was affected by the intracellular free Ca. The effect, therefore, can be analysed by comparing the maximum inward Na and Ca current. However, the maximum inward Na current also varies depending upon the intracellular Na concentration or the Na

TABLE 4. Na and Ca currents with various internal Ca concentrations

Egg no.	Ext. soln. (mM)	Int. soln. (mM)	t_d (min)	$E_{r.Na}$ (mV)	$I_{h\text{-}90}$ (nA)	Na current $V_{\frac{1}{2}}$ (mV)	Na current $V_{p.max}$ (mV)	Na current $I_{p.max}$ (10 nA)	Na current G_0 (μmho)	Ca current $V_{\frac{1}{2}}$ (mV)	Ca current $V^{(Ca)}_{p.max}$ (mV)	Ca current $I^{(Ca)}_{p.max}$ (nA)
					(OCa with 50 mM-GEDTA and 50 mM-DPTA-OH)							
77031511	400 Na	400 Na										
	100 Ca(α)	170 Cl	6·5	20	0·5	—	−21·5	2·13	0·74*	+6	23	0·92*
77050801	400 Na	400 Na										
	100 Ca	170 Cl	4	23	0	−33	−18	1·86	0·52	+8	28	2·9
77050806	400 Na	400 Na										
	100 Ca	170 Cl	6	23·5	0·2	−34	−20	1·27	0·38	+8	26	2·9
Average				22·2		−33·5	−19·8			+7·3	25·7	
					(45 Ca $1·8 \times 10^{-6}$ M)							
77050809	400 Na	400 Na										
	100 Ca	170 Cl	4–8	19	0·3	−35	−21	1·67	0·53	+9·5	25	2·9
77050810	400 Na	400 Na										
	100 Ca	170 Cl	6	29	0·5	−34	−20	1·73	0·45	+8	25	2·4
Average				24		−34·5	−20·5			+8·8	25	
					(57 Ca $3·3 \times 10^{-5}$ M)							
77050804	400 Na	400 Na										
	100 Ca	170 Cl	6	21	0·5	−33	−21	2·48	0·78	+11	25	1·2
					(60 Ca 5×10^{-5} M)							
77031512	400 Na	400 Na										
	100 Ca	170 Cl	7	30	2·3	—	—	—	0·73	+7	24	1·2
Total average				23·6		−33·8	−20·3			+8·2	25·1	

* The egg showed low Ca current less than 1·0 nA with zero Ca perfusate.

TABLE 5A. Effects of internal Ca^{2+} on Na and Ca currents

Egg no.	Ext. soln. (mM)	Int. soln. (mM)	Buffer (mM)	$[Ca^{2+}]_i$ (M)	t_d (min)	$E_{r.Na}$ (mV)	$I_{h\text{-}90}$ (nA)	G_0(Na) (μmho)	$V^{(Ca)}_{p.max}$ (mV)	$I^{(Ca)}_{p.max}$ (nA)
77030401	Solution α	170 Cl(D)	100 EG*	0	5·5	25·5	1·0	0·42	17·5	2·5
77030402	Solution α	170 Cl(D)	100 EG	0	2·5	25	0·7	0·57	15	1·9
77030404	Solution α	170 Cl(D)	100 EG	0	2·5	40	1·0	0·56	17·5	*0·6*
77031213	Solution α	170 Cl(D)	100 DP†	0	2	36	0·8	0·97	18	*0·6*
77031504	Solution α	145 Cl(G)	50, 50‡	0	3	30·5	1·0	0·87	16·5	*0·4*
77031505	Solution α	145 Cl(G)	50, 50	0	6	31	0·3	0·36	25·5	2·6
77031508	Solution α	145 Cl(G)	50, 50	0	4·5	40	0·6	0·47	23	2·7
77031511	Solution α	145 Cl(G)	50, 50	0	4	28·5	0·6	0·84	22·5	*0·9*
77031513	Solution α	145 Cl(G)	50, 50	0	3	29·5	1·6	0·50	26	2·1
77050801	Solution α	100 Cl(I)	50, 50	0	3	30·5	0	0·49	28	2·9
77050806	Solution α	100 Cl(J)	50, 50	0	3·5	36·5	0·2	0·37	26	2·9
Average						32 ± 5				
77030403	Solution α	120 Cl(F)	100 EG 80 Ca§	8×10^{-7}	2	38·5	0·8	0·57	18·5	*0·8*
77050808	Solution α	100 Cl(J)	50, 50 45 Ca	$1·8 \times 10^{-6}$	4	39	0·8	0·80	25	1·8
77050809	Solution α	100 Cl(J)	50, 50 45 Ca	$1·8 \times 10^{-6}$	4	34·5	0·1	0·45	25	2·9
77050810	Solution α	100 Cl(J)	50, 50 45 Ca	$1·8 \times 10^{-6}$	6	29	0·5	0·45	25	2·4
77050803	Solution α	100 Cl(J)	50, 50 57 Ca	$3·3 \times 10^{-5}$	3·2	41	0·5	0·81	17	1·4
77050804	Solution α	100 Cl(J)	50, 50 57 Ca	$3·3 \times 10^{-5}$	4	32	0·5	0·91	25	1·2
77031512	Solution α	145 Cl(H)	50, 50 60 Ca	5×10^{-5}	7	30	2·3	0·73	26·5	1·0
77031211	Solution α	120 Cl(F)	100 DP 80 Ca	8×10^{-4}	2	42·5	0·3	0·74	16·5	0·2
77031212	Solution α	120 Cl(F)	100 DP 80 Ca	8×10^{-4}	5	28·5	2·0	0·77	16	0·2
77031506	Solution α	145 Cl(G)	50, 50 90 Ca	8×10^{-4}	10	29	2·0	0·75	26·5	0·8
77031507	Solution α	145 Cl(G)	50, 50 90 Ca	8×10^{-4}	5·5	28	0·5	0·86	26	0·8
77031509	Solution α	145 Cl(G)	50, 50 90 Ca	8×10^{-4}	1·2	(40)	0·7	0·90	17·5	0·7
Average						34 ± 5				

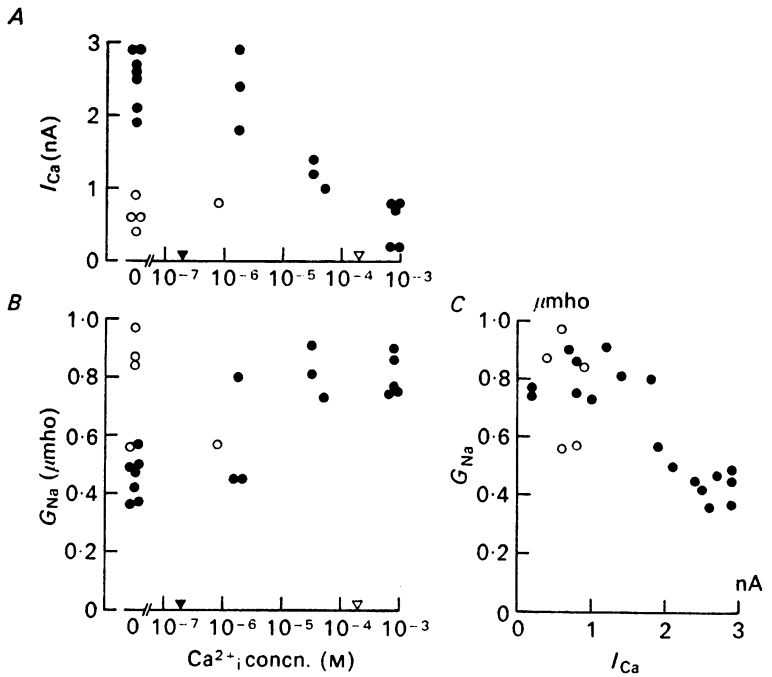
* GEDTA. † DPTA-OH. ‡ 50 mM-GEDTA and 50 mM-DPTA-OH. § 80 mM-$Ca(OH)_2$ added.

TABLE 5*B*. Effects of internal Ca^{2+} on Na and Sr currents

Egg no.	Ext. soln. (mM)	Int. soln. (mM)	Buffer (mM)	$[Ca^{2+}]_i$ (M)	t_d (min)	$E_{r.Na}$ (mV)	$I_{h.90}$ (nA)	G_0(Na) (μmho)	$V^{(Ca)}_{p.max}$ (mV)	$I^{(Ca)}_{p.max}$ (nA)
77011403	Solution β	170 MS(L)	100 EG	0	2·5	28	1·5	0·41	16·5	2·2
77011404	Solution β	170 MS(L)	100 EG	0	1·5	38	0·3	0·27	5	6·6
77011405	Solution β	170 MS(L)	100 EG	0	1·5	36	0·3	0·24	15	4·1
77011406	Solution β	170 MS(L)	100 EG	0	1·8	40	0·2	0·24	15	4·4
77011407	Solution β	170 MS(L)	100 EG	0	5	21	1·0	0·35	15	3·4
77011410	Solution β	170 MS(L)	100 EG	0	8	26	0·15	0·35	15	3·8
77020103	Solution β	170 MS(L)	100 EG	0	2	24·5	0·5	0·23	16	2·8
77020104	Solution β	170 MS(L)	100 EG	0	3·5	25	0·15	0·32	8	4·6
77020105	Solution β	170 MS(L)	100 EG	0	5	27	0·3	0·27	17	4·2
88020110	Solution β	170 MS(L)	100 EG	0	8	25	2·0	0·36	9	2·8
77020111	Solution β	170 MS(L)	100 EG	0	4·5	21	0·15	0·23	20	1·2
Average						28 ± 6		0·30 ± 0·06		3·6 ± 1·4
77011408	Solution β	170 MS(M)	100 EG 90 Ca	$1·8 \times 10^{-6}$	2·5	23·5	0·8	0·57	16	1·2
77011409	Solution β	170 MS(M)	100 EG 90 Ca	$1·8 \times 10^{-6}$	1·5	39·5	0·5	0·62	16	0·6
77020106	Solution β	170 MS(M)	100 EG 90 Ca	$1·8 \times 10^{-6}$	6·5	31	1·0	0·51	9	2·0
77020107	Solution β	170 MS(M)	100 EG 90 Ca	$1·8 \times 10^{-6}$	4·5	23	0·4	0·51	8	2·2
77020108	Solution β	170 MS(M)	100 EG 90 Ca	$1·8 \times 10^{-6}$	5·5	27	0·2	0·52	9	2·0
Average						29 ± 6		0·55 ± 0·04		1·6 ± 0·3

reversal potential, while the maximum Ca current is independent of the Na reversal potential, as described before. Thus, for Na channels, the zero-current conductance was also used for the indicator in the intracellular Ca experiment as in the F^- or Cl^- experiment.

Text-fig. 8*A* and *B* show collected data of the maximum Ca current (I_{Ca}) and the zero-current Na conductance (G_{Na}) from twenty-three egg cells which were perfused with different solutions containing various free Ca concentrations. The measurements were all done when the Na reversal potential was within the range from +25 to +40 mV to minimize the effect of intracellular Na concentration ($[Na]_i$)



Text-fig. 8. The relationships of peak Ca current I_{Ca} (*A*) and zero current Na conductance G_{Na} (*B*) when intracellular free Ca concentrations were determined by various Ca buffer solutions. Each point in the respective figures was obtained from an egg preparation. A total of twenty-three eggs were illustrated. The data are all listed in Table 5*A*. Filled and open triangles on the abscissa indicate apparent binding constants for GEDTA and DPTA-OH at pH 7·0 respectively. Open circles indicate the egg preparations which showed small I_{Ca} less than 1·0 nA. *C* demonstrates the reciprocal relationship between I_{Ca} (abscissa) and G_{Na} (ordinate). Each point was obtained from an egg. The open circles have the same meaning as in *A* and *B*.

upon G_{Na}. Since the difference in E_r, 15 mV, corresponds to 1·8 times change in $[Na]_i$ and G_{Na} is roughly proportional to the square root of $[Na]_i$, the effect of $[Na]_i$ upon G_{Na} will be kept within a factor of 1·3. The external solution α containing 100 mM-Ca was used for all eggs examined. All experimental data on these eggs are listed in Table 5*A*.

In Text-fig. 8*A* and *B*, the I_{Ca} and G_{Na} were plotted against the Ca ion concentration in the buffered perfusate. The results showed that I_{Ca} which was 2–3 nA in the zero Ca perfusate decreased as the Ca concentration was increased above 10^{-6} M

and became one fifth at 8×10^{-4} M. By contrast, G_{Na} began to increase at Ca concentration above 10^{-6} M and became maximum around 5×10^{-5}–10^{-4} M. The G_{Na} became about twice that of the zero Ca solution. G_{Na} seemed to decrease slightly above 10^{-4} M-Ca. Therefore, it is concluded that the intracellular free Ca ions in the range from 10^{-6} to 10^{-3} M have a reciprocal effect upon Na and Ca channels.

The open circles in Text-fig. 8*A* and *B* indicate the data from the eggs which showed a small I_{Ca} less than 1 nA in zero Ca or 8×10^{-7} M-Ca perfusate. These eggs always showed a relatively large G_{Na} in comparison with the eggs which showed I_{Ca} of 2–3 nA. Although there was no definite evidence, it is possible that those eggs which showed the small I_{Ca} may have had a higher internal concentration of free Ca, possibly because of an increased Ca influx through non-specific leakage. In a few experiments, in which the internal perfusate contained more than 10 mM-Ca ions and no chelating agents, both Na and Ca currents were abolished irreversibly.

In Text-fig. 8*C*, the G_{Na} was plotted against the I_{Ca} of the same egg perfused with various concentrations of intracellular free Ca, from the same data illustrated in Text-fig. 8*A* and *B*. There is a clear reciprocal relationship between G_{Na} and I_{Ca}. It is especially interesting that the eggs which showed a relatively low Ca current, even during the perfusion of low intracellular free Ca, tended to have a larger G_{Na}, as indicated by open circles in Text-fig. 8*C*.

The effects of intracellular Ca upon Na and Sr currents were also examined by using the external solution β and internal solution containing methanesulfonate (solution K or M). The results are listed in Table 5*B*. In the internal methanesulphonate perfusate of zero free Ca, G_{Na} was 0·30 μmho which was slightly less than that in the internal Cl perfusate and the maximum Sr current I_{Sr} was 3·6 nA, while, in the internal perfusate of $1{\cdot}8 \times 10^{-6}$ M-Ca, G_{Na} increased to 0·55 μmho and I_{Sr} decreased to 1·6 nA. The reciprocal effect of internal Ca ions on Na and Ca channels was again confirmed.

DISCUSSION

In the large axons successful studies have been carried out using intracellular perfusion (Baker, Hodgkin & Shaw, 1962). However, the application of this technique to more spherical cells has been hampered by the difficulty of keeping the membrane intact after making a hole in it. In the present study the egg cell of the tunicate was successfully perfused intracellularly by a method whose principle is similar to that described by Kostyuk *et al.* (1975) and Lee *et al.* (1977). In our experiment, however, the cleaned surface of the egg was brought into contact with a smooth Pyrex glass wall inside a small funnel, instead of using a hole in the polyethylene sheet or a suction pipette. Close contact was facilitated by coating a positively charged protein, protamine, on the glass wall. The intact membrane free from this contact was estimated as half of the total surface of the intact egg and used for the experiment of the internal perfusion. Accordingly, the peak Na and Ca currents were half compared to those found in the intact egg, and the leakage current also became half. The free membrane appeared to behave just the same as in the intact egg, despite the fact that the membrane on the opposite side had a hole almost a quarter of the egg diameter. The degree of exchange of the intracellular solution, monitored by the shift in the reversal potential for Na current, was found to be 80–95% complete within 30 min.

In the present experiment, the selectivity ratios for the Na channels were found to be $P_{Na}:P_K:P_{Rb}:P_{Cs} = 1{\cdot}0:0{\cdot}14:0{\cdot}05:0{\cdot}04$ by changing cationic composition of the internal perfusate. The ratios obtained were essentially the same as those deduced in the intact egg cell by varying the composition of the external solution (Okamoto *et al.* 1976*a*). The ratios were also similar to those obtained for the Na channels of other excitable membranes (Hille, 1970; Campbell, 1976; Table III in Ebert & Coldman, 1976). These results confirm the previous suggestions (Okamoto *et al.* 1976*a*, *b*) that the egg Na channel is identical with those found in the other excitable membranes and further suggest that the permeation mechanism of the Na channels was grossly unchanged with the artificial internal perfusate containing chelating agents and fluoride ions.

Internal perfusates containing F^- ions were found to be the best for reducing the leakage current and keeping the amplitude of Na current large. This effect of F^- ions is well known for squid axons, *Myxicola* axons and snail neurones (Tasaki *et al.* 1965; Ebert & Goldman, 1976; Kostyuk *et al.* 1975). In the tunicate egg, the internal F^- ions not only enhanced the Na current but also shifted its current–voltage relation along the voltage axis in a negative direction as compared with the internal Cl^- ions. It may be inferred that the increase in the Na conductance is derived from the lowered threshold for Na channel-gating. According to the theory of the diffuse double layer (Gilbert, 1971) a negative shift in the current–voltage relation corresponds either to an increase in the negative charges or the negative surface potential at the outer surface or to a decrease of them at the inner surface. Thus, in order to explain the present results in terms of a change in the surface potential, one would have to conclude that there is a specific binding to inner membrane by Cl^- ions and not by F^- ions or alternatively, that Cl^- ions somehow extract a positively charged substance from the internal membrane surface, this substance being unaffected by F^- ions. In the tunicate egg, 170 mM-F^- ions in perfusate abolished Ca or Sr current completely whereas they doubled Na current. This suppressive effect of internal F^- ions upon Ca current has also been found in snail neurones (Kostyuk *et al.* 1975). The reciprocal effect on Na and Ca currents can hardly be explained only by the uniform changes in the surface potential at either side of the membrane. It is therefore premature to draw a conclusion about the mechanism of the fluoride effects on the egg membrane.

The internal free Ca concentration ($[Ca^{2+}]_i$) of more than 10^{-6} M was inhibitory for Ca spike generation in barnacle muscle fibres (Hagiwara & Nakajima, 1966). Kostyuk *et al.* have reported that $[Ca^{2+}]_i$ over 2×10^{-8} M abolish Ca current in perfused snail neurones (Kostyuk & Krishtal 1977). The present experiments indicated that, at a $[Ca^{2+}]_i$ greater than 10^{-6} M, a reduction of Ca current occurred and the complete abolition would probably require $[Ca^{2+}]_i$ more than 1 mM. It seems, therefore, that the egg membrane is slightly less sensitive to the internal Ca ions than the barnacle muscle and considerably less than the snail neurone, which might be of some developmental significance in view of the undifferentiated stage of the egg membrane. However, the recent studies on Ca-accumulating and releasing structures, such as the sarcoplasmic reticulum, reveal the existence of Ca-induced Ca release inside of the cell (Ford & Podolsky, 1970; Thorens & Endo, 1975). If this mechanism is also applied to the barnacle muscle and the snail neurone, the intracellular Ca in

these experiments might have been much higher than that prescribed by the buffer solution used. Since, in the present experiments, high concentrations of Ca chelating agents as well as pH buffers were used (see Methods), the unregulated rise in the Ca ion concentration was less likely to occur. However, very localized enhancement of Ca ion concentration would still be possible inside the egg cell (Ridgway *et al.* 1977).

A facilitatory effect of internal Ca ions upon the Na current has not previously been found. Moreover, there have been a few reports which suggest that there is no effect or rather suppressive effect of internal Ca ions up to 10 mM in squid giant axons (Tasaki, Watanabe & Lerman, 1967; Begenisich & Lynch, 1974), although Begenisich & Lynch (1974) have reported an enhancement of Na current in one axon of the squid perfused with 10 mM-Ca solution. Yamagishi (1977) has also suggested the possibility of increase in Na permeability with risen intracellular Ca ions in squid giant axons. Since the experiments on the effect of internal Ca ions upon Na channels cannot be carried out by using F^--perfused axons because of the precipitation of CaF_2, the decreasing tendency of Na current may be inevitable in Cl^--perfused axons, as we also observed for the egg cells. This degeneration of Na current may obscure the enhancement of Na current induced by high internal Ca ions.

Since the kinetic properties or gating mechanisms of the Na channels were almost unaffected by the intracellular Ca ions, the enhancement of the Na current must be derived from an increase in the total number of the channels if the unit conductance of each Na channel is not changed. On the analogy of ACh receptors on the developing muscle membrane (Devreotes & Fambrough, 1975), an increase in the membrane-bound molecules, such as Na channel proteins, may be induced by one or two out of the following four causes: (1) increased synthesis of the membrane protein, (2) insertion of the membrane protein from the intracellular sites into the surface plasma membrane, (3) activation of the inactivate form of the protein molecule or conversion of a precursor into the functional molecule, and (4) inhibition of the degeneration mechanism for the membrane protein, the first, third, and fourth causes have been suggested to required the metabolic energy and may have a time lag until the changes in the distribution of the molecules actually occur on the plasma membrane. Therefore, it is unlikely for these three causes to be responsible for the enhancement of Na conductance, because of the abrupt increase within a few minutes after increasing the intracellular free Ca ions. Considering the fact that the replacement of Cl^- with F^- in the internal perfusate also doubled Na conductance, it may be suggested that there are immediately available reserves of Na channels in the intracellular membranous structures and that the channel molecules are in a state of equilibrium between the intracellular structures and the surface plasma membrane. Then, some agents, such as intracellular free Ca ions or fluoride ions, may shift the equilibrium toward the plasma membrane side, assuming that the initial number of the reserves is comparable to that of the Na channels in the surface membrane. It may further be possible that the Ca channels themselves are the reserves of the Na channels because the effects of free Ca on F^- ions were reciprocal. However, the abolition of Ca current by intracellular high free Ca is not accompanied by the enhancement of Na current in the barnacle muscle membrane where no Na component has been observed (Hagiwara & Nakajima, 1966), and the enhancement of Na current by internal F^- ions is not followed by the abolishment of Ca current in the squid axons where very few

Ca channels exist (Tasaki *et al.* 1967). Thus, direct interconversion between Na and Ca channels seems unlikely to occur in the case of the egg cell membrane as well.

The reciprocal effect of the internal free Ca ions upon Na and Ca currents in the egg membrane are very interesting for formulating the roles of Ca ions on development and differentiation as well as for describing the essential properties of the egg membrane as an excitable membrane. In a cytochalasin B-arrested tunicate embryo it is observed that the Ca channels found in the egg membrane are segregated into the presumptive muscle cells while Na channels are accumulated in ectodermal, probably presumptive nerve cells (K. Takahashi and M. Yoshii unpublished results). Therefore, initial existence of both Na and Ca channels in the egg membrane will require the differential effect of some intracellular factors upon the distribution and segregation of each kind of the channels during development of the excitable membranes in the embryo. There are also several reports that Ca channels with or without Na channels exist in the less differentiated states of excitable cells, such as myotubules (Kidokoro, 1975; Kano & Yamamoto, 1977), Rohon Beard cells (Spitzer & Baccaglini, 1976), and neuroblastoma cells (Spector, Kimhi & Nelson, 1973; M. Miyake, personal communication), and that the pure Na spikes appear only in the fully differentiated state. The results of the present experiment suggest that an alternation in the intracellular free Ca ions is capable of producing a differential effect upon the distribution of Na and Ca channels and that this may be one of the variables used to bring about these forms of membrane differentiation.

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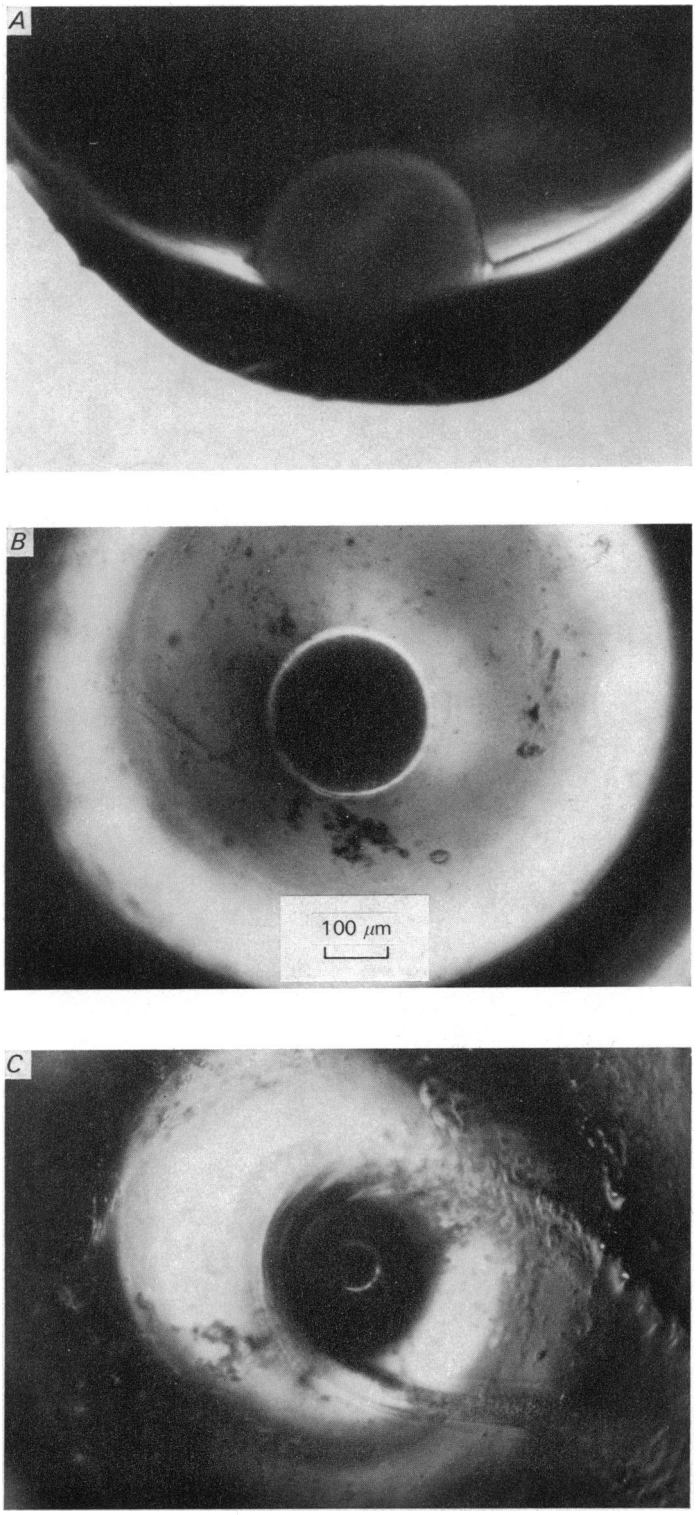
A
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EXPLANATION OF PLATE

The photographs of an egg inside the small glass funnel (see Methods). *A* is a side view. The lower membrane was ruptured at the lower orifice of the funnel. *B* is top view. *C* is bottom view, the ruptured hole of the membrane or the lower orifice of the funnel being 80 μm in diameter. Scale is 100 μm in *B*. In *A* the egg seems to be photographed in the more expanded scale probably due to the lens effect of the glass wall of the funnel.