Biochimica et Biophysica Acta, 599 (1980) 623—638

BBA 78774

NEUTRAL CARRIER ION-SELECTIVE MICROELECTRODES FOR MEASUREMENT OF INTRACELLULAR FREE CALCIUM

R.Y. TSIEN and T.J. RINK

Physiological Laboratory, University of Cambridge, Cambridge, CB2 3EG (U.K.)

(Received August 23rd, 1979)

Key words: Intracellular Ca^{2+}; Neutral carrier; Ion-selective electrode; Siliconization; Ca^{2+} buffer

Summary

This paper describes the making and testing of calcium-selective microelectrodes for measurement of intracellular [free Ca^{2+}] levels. Pipettes of tip outer diameter down to 0.4 μm were siliconized by a novel and easy method of vapor treatment. The tips were filled with a sensor mixture using the neutral ligand and solvent of Oehme et al. (Oehme, M., Kessler, M. and Simon, W. (1976) Chimia (Aarau) 30, 204—206) but with very hydrophobic cations replacing Na^+ in the salt component. This change improved electrode stability and greatly reduced hysteresis in the responses to changing [Ca^{2+}] levels. Lowering the Ca^{2+} concentration in the internal electrolyte also increased electrode lifetime.

Electrodes showed a Nernstian response to [Ca^{2+}] down to 1 μM free concentration in 0.1 M KCl, and usually a useful response to below 100 nM Ca^{2+}. Selectivity for Ca^{2+} over Mg^{2+} and H^+ was sufficiently high that typical free intracellular levels of Mg^{2+} and H^+ caused negligible interference. Selectivity for Ca^{2+} over Na^+ was adequate for cells with 10^{-2} M free Na^+, but higher levels could raise significantly the detection limit for Ca^{2+}. Preliminary measurements of [free Ca^{2+}] have been made in frog skeletal muscle, ferret ventricular myocardium, and early embryos of *Xenopus laevis*. Relative merits of Ca^{2+} microelectrodes and optical indicators are discussed.

Abbreviations: BPh_4, tetraphenylborate; e.m.f., electromotive force or potential between the ion-sensitive electrode and a reference electrode in the same solution; *o*-NPOE, (*o*-nitrophenyl)octylether; EGTA, ethyleneglycolbis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; HEEDTA, N-(2-hydroxyethyl)ethylenediamine-*N,N',N'*-triacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); Taps, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid.

Introduction

Changes in cytoplasmic free Ca^{2+} concentration are implicated in an increasing variety of cellular functions [2,3], and techniques are needed for monitoring [free Ca^{2+}] levels in intact cells [4]. Such measurement is technically demanding for many reasons, including the small size of most cells and the low levels of [free Ca^{2+}] to be measured in the presence of high levels of potentially interfering cations. Thus the cytoplasmic resting [free Ca^{2+}] level may be 10^{-7} M, whereas typical values for other cations would be 10^{-7} M H^+, 10^{-3} M Mg^{2+}, 10^{-2} M Na^+, and 10^{-1} M K^+. Thus any system for monitoring [free Ca^{2+}] must have very high selectivity. At present the three main methods use the photoproteins aequorin and obelin, Ca^{2+}-indicator dyes, and ion-selective microelectrodes. While intracellular electrodes selective for Na^+, K^+, H^+ and Cl^- are well documented [5] and widely used, only recently have Ca^{2+} electrodes been made with selectivities even approaching the necessary levels. Two classes have been reported: (1) those based on organophosphate anions [6—8], which appear to have inadequate selectivity against Mg^{2+} for intracellular use, although their selectivity against Na^+ and K^+ is excellent; (2) electrodes using a neutral ligand. The initial report [1] on such microelectrodes showed selectivities which would be insufficient for detecting intracellular [Ca^{2+}] levels, but more recently there have been preliminary reports of versions with much better performance [9,10], satisfactory for intracellular use. However those electrodes had tip diameters above 1 μm, and application was limited to large cells.

This paper reports our experience with neutral ligand Ca^{2+}-selective microelectrodes, problems that have arisen, and steps taken to overcome them, so that electrodes with responses adequate for intracellular use and tip size down to 0.4 μm can be produced.

Methods

Sensor compositions

The sensor initially used was a gift from Professor W. Simon of the mixture according to Oehme et al. [1]: 10% of the neutral ligand *N,N'*-di(11-ethoxycarbonyl)undecyl-*N,N'*,4,5-tetramethyl-3,6-dioxaoctane-1,8-dioamide and 1% sodium tetraphenylborate ($NaBPh_4$) in (*o*-nitrophenyl)octylether (*o*-NPOE). For studies on modifying the sensor composition, the neutral ligand was synthesized following the method of Ammann et al. [11] except that final purification was by chromatography with $CHCl_3/CH_3OH$ on silica gel instead of benzene/acetone. The resulting material was identical with a small sample kindly provided by Professor Simon as judged by TLC, NMR and performance in electrodes. Most subsequent studies were done with a 'red sensor' made from 10% of the neutral ligand and 2.4% tetraphenylphosphonium bis(1,3-diethyl-2-thiobarbiturate)trimethine oxonol, in *o*-NPOE. The oxonol salt was prepared by precipitating the Tris salt of the oxonol dye [12] with a solution of tetraphenylphosphonium chloride. The oxonol dye imparts a brilliant ruby red color to the mixture, hence the name.

Fabrication of electrodes

Pyrex capillary tubing (GC150T, Clarke Electromedical, Pangbourne,

Reading, U.K.) of outer diameter 1.5 mm and inner diameter 1.0 mm is cleaned by partial immersion in 55% HNO_3 and addition of small aliquots of C_2H_5OH at intervals to maintain a vigorous but controlled evolution of NO_2 (caution: the reaction beaker should be less than one tenth full and be placed in an efficient hood). The glass is then rinsed and finally boiled in multiple changes of distilled water. After drying, the tubes are drawn into micropipettes on a conventional automatic horizontal puller. The extreme tips of the micropipettes are broken under microscopic observation by butting them against the edge of a microscope slide advanced by a micromanipulator. The resulting tip outer diameter is in the range 0.3—0.8 μm; the very gentle taper just behind the tip is the reason for breaking back a fine micropipette rather than setting the puller itself to produce the requisite tip diameter. The tips are routinely examined with a 0.65 NA * objective at 625× magnification against a calibrated eyepiece graticule. Tip size measurements seem to be accurate to ±0.2 μm or better [13] as judged by comparison with high power scanning electron micrographs. The pipettes are then inserted, tips upward, into holes drilled into a small aluminum block. When a convenient number (10—25) have been accumulated, the block and pipettes are placed in a glass crystallizing dish in an oven at 200°C. After approx. 30 min at that temperature to drive off water adsorbed onto the glass [14], 10 μl of pure tri-*n*-butylchlorosilane (Pfaltz and Bauer, Stamford, CT U.S.A.) are dropped onto the floor of the dish alongside the block. A loose-fitting lid (a petri dish preheated to 200°C) is then put on the dish. The silane vapor is allowed approx. 15 min to react with the glass, then the lid is removed for another 15 min to allow excess silane to disperse. Only then are the dish, block, and pipettes taken out of the oven to cool. Siliconized pipettes may be stored without deterioration for many days in the dish with the lid on. Individual pipettes are taken out as required and filled by syringe needle from the shoulder backwards with the p*Ca* 7 buffer solution described in Table I. Air pressure is applied from another syringe via flexible tubing to force the electrolyte into the pipette tip, expelling the air in front of it. The electrode may now be beveled, for example on a commercial abrasive turntable device (W-P Instruments, New Haven, CT, U.S.A.); typically the beveling is continued until the initial electrode resistance is halved. The ability to monitor beveling by the fall in resistance is one reason for putting electrolyte into the micropipette before introducing the sensor. A second reason is that debris from the abrasion can be dislodged by expelling electrolyte or sucking in sensor. A third advantage is that the electrode without sensor can be inserted into the preparation to check that it records healthy membrane potentials; if it does not, then a sharper electrode is required. The electrode is then reconnected to the syringe and mounted with plasticine onto the arm of a micromanipulator. Under observation through a dissecting microscope, the electrode tip is steered into a droplet of sensor held in a fire-polished and necked-down glass capillary. A column of sensor is sucked into the electrode by partial vacuum from the

* NA, numerical aperture.

TABLE I

COMPOSITION OF ROUTINE CALIBRATING SOLUTIONS

Each different p*Ca* solution contained 10 mM of the specified pH buffer and 10 mM total Ca^{2+} ligand except for p*Ca* 3 which contained no ligand. The pH was brought to the specified level by titration with KOH. It will be noted that the final Cl^- concentration is always 100 mM. NTA, nitrilotriacetic acid.

p*Ca*	[$CaCl_2$] (mM)	Ca^{2+}-ligand	[KCl] (mM)	pH buffer	pH
3	1	none	98	Mops	7.30
4	5	NTA	90	Hepes	7.39
5	5	NTA	90	Taps	8.42
6	5	HEEDTA	90	Hepes	7.70
7	5	EGTA	90	Mops	7.29
8	5	EGTA	90	Hepes	7.80
'∞'	0	EGTA	100	Hepes	7.80

syringe. When enough sensor has been taken up, first the vacuum is released by disconnecting the syringe, then the electrode tip is withdrawn from the droplet. Finally the shank of the electrode is painted to within approx. 0.5 mm of the tip with silver conductive paint (Electrodag 416, Acheson Colloids Co., Port Huron, MI, U.S.A.), placed so that the bathing solution level will reach midway up the painted zone, in order to reduce the capacitive artifacts produced by fluctuations in the solution level.

Electrode testing and calibration procedure

Calcium electrodes are calibrated in solutions held in a small glass funnel the stem of which is bent to form an N-shaped siphon trap. The funnel is held by plasticine to the inner wall of a beaker inside a Faraday cage. The calcium electrode is lowered into the funnel so that the solution level reaches halfway up the silver-painted zone. The reference electrode is a fine plastic catheter filled with 3 M KCl and inserted into the outlet of the siphon trap. The other end of the catheter connects to a disposable syringe with a chloridized silver wire passing through the rubber plunger. The bathing solution is changed by inserting a Pasteur pipette through a small hole in the Faraday cage and gently squirting 1 ml of the new solution onto the sloping wall of the funnel. The solution change is complete in less than 1 s. If the new solution has a grossly different ionic background from the old, fresh KCl should be extruded from the reference catheter to renew an abrupt liquid junction. The e.m.f. between the calcium electrode and the reference electrode is measured with an electrometer with an Analog Devices (Norwood, MA, U.S.A.) 42K operational amplifier as input buffer. Also incorporated is a circuit through which ±0.1 to 1000 pA can be injected into the electrode to permit measurement of resistance. E.m.f. is displayed on a digital voltmeter and recorded on a chart recorder.

Solutions

The compositions of the routine calibrating solutions are given in Table I. Except for the p*Ca* 3 and '∞' solutions, the free Ca^{2+} concentrations are buffered with 10 mM ligand to 5 mM Ca^{2+}. The 1 : 1 ratio of free ligand : Ca^{2+}-

bound ligand maximizes the buffering power. The desired free Ca^{2+} concentration is achieved by choice of ligand and appropriate adjustment of the solution pH. This procedure is justified because the electrodes are virtually insensitive to pH changes over the range used in the calibration solutions. All solutions contain 100 mM KCl plus the K^+ salts of the buffers. This ionic background was chosen because: (1) the K^+ concentrations and ionic strengths are reasonably close to those inside vertebrate cells; (2) the published stability constants [15] were determined in 100 mM KCl.

A comment is necessary on the calculation of apparent Ca^{2+} stability constants from the tabulated absolute constants. Martell and Smith [15] note that the usual figures for proton ionization constants must be corrected upwards by 0.11 units when working in 0.1 M ionic strength. This correction seems to have been generally ignored in the biological literature. It arises because the chemists who determined the constants defined the pK_a values using the concentration, not the activity, of the hydrogen ion. For example, the usual figure of 9.46 for the highest pK_a of EGTA [16] means that $[EGTA^{4-}]\ [H^+]/[H \cdot EGTA^{3-}] = 10^{-9.46}$ M, where all bracketed terms represent concentrations in 100 mM KCl. However, pH is defined as $-\log(a_H)$ where by National Bureau of Standards convention a_H is 0.78 $[H^+]$ in 0.1 M ionic strength. Therefore $[EGTA^{4-}]/[H \cdot EGTA^{3-}] = 10^{(pH-9.57)}$, as discussed by Boyd et al. [17]. The net effect of the p*Ka* correction is to lower the estimates of the apparent Ca^{2+} affinities of EDTA, HEEDTA, and nitrilotriacetic acid by approx. 0.11 log unit and that of EGTA by approx. 0.22 log unit at physiological pH.

The [free Ca^{2+}] levels and p*Ca* values cited refer to Ca^{2+} concentrations in 0.1 M ionic strength, as customarily understood in Ca^{2+}-buffer calculations. The activity coefficient for Ca^{2+} is practically constant for $[Ca^{2+}] < 1$ mM because of the swamping effect of the 100 mM KCl. Our convention is to define the standard state for $[Ca^{2+}]$ to be infinite dilution into 0.1 M KCl. This makes the activity coefficient simply 1, fits the usual convention for Ca^{2+} buffers, and is realistic for the intracellular medium, which is almost always at a constant ionic strength regardless of $[Ca^{2+}]$ levels. Other authors [11,18] have chosen to calculate the Ca^{2+} activity coefficient from Debye-Huckel theory, thus taking the standard state for $[Ca^{2+}]$ to be infinite dilution into distilled water. The latter convention assigns to our solutions a p*Ca* value approx. 0.41 to 0.45 higher than our definition for p*Ca*. For example, our p*Ca* 7 solution would be designated p*Ca* 7.41—7.45, so that the calibration plots would look more impressive.

Results and Discussion

In our hands the performance of Ca^{2+}-selective microelectrodes is quite variable, even between electrodes made in the same batch and as far as possible in the same way. Each electrode must be tested individually for response to $[Ca^{2+}]$ levels below 10 μM. Fig. 1 shows calibration curves for two microelectrodes made with sensor of the original formulation [1]. Fig. 1A gives the calibration plot for one of the best electrodes of this type, which gave a virtually Nernstian response down to p*Ca* 7 and a further useful response down to p*Ca* 8 and lower. In contrast, Fig. 1B illustrates two defects sometimes seen.

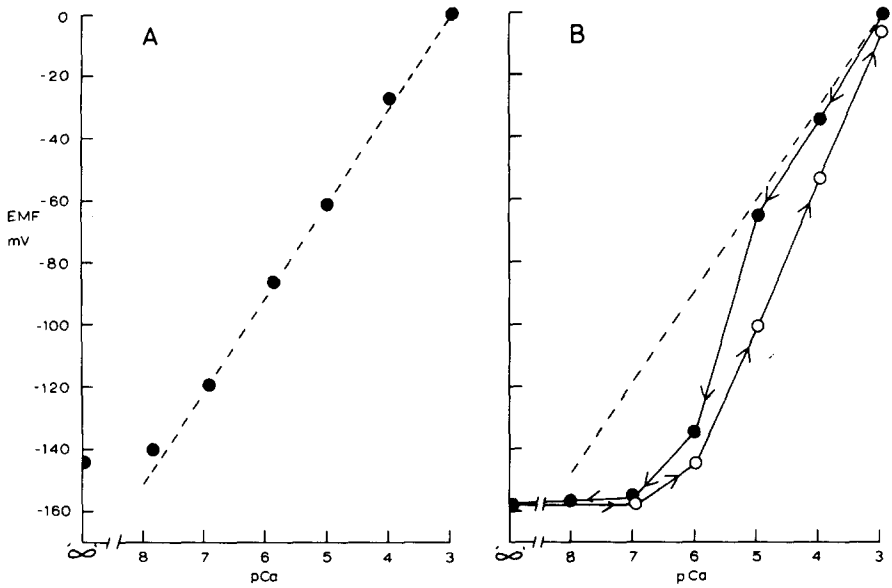

Fig. 1. Calibration plots for two electrodes made with $NaBPh_4$ sensor. A. 1 μm tip electrode. Plotted is the e.m.f. response to progressively lower free Ca^{2+} concentrations. This electrode showed little hysteresis. B. Another 1 μm electrode. The filled circles show the response to successively decreasing free Ca^{2+} concentrations and the open circles to increasing concentrations. The composition of the calibration solutions is given in Table I. The dashed lines show the theoretical (Nernstian) response for $[Ca^{2+}]$. The e.m.f. has been arbitrarily offset to give 0 mV in p*Ca* 3.

The calibration slope is greater than Nernstian going from p*Ca* 5 to 7 and also returning from p*Ca* 6 to 3. This electrode also showed hysteresis in that the actual potentials reached were substantially lower in the calibration ascending in $[Ca^{2+}]$. Such hysteresis was rather common with sensors containing $NaBPh_4$ and was usually most prominent between p*Ca* 8 and p*Ca* 5. The failure to respond much to elevation of free Ca^{2+} in this range would prevent the observation of transient elevations of intracellular $[Ca^{2+}]$, which is frequently the effect one is trying to measure. Another feature of electrodes made with $NaBPh_4$ was that the responses to changes in $[Ca^{2+}]$ below 10 μM were often very slow, sometimes taking minutes to reach a new steady potential. The response usually had two clearly distinct phases — approx. two-thirds of the final excursion being complete in a few seconds and the remainder taking many times longer.

Electrodes made with sensor containing lipophilic cations

As explained in the Appendix, the undesirable hysteresis and two-phase time course were tentatively blamed on the loading of an exchangeable cation, Na^+, into the sensor. Electrodes were therefore made with a sensor in which tetraphenylphosphonium replaced Na^+ mole for mole. Unfortunately, the original anion, tetraphenylborate, could not be retained because tetraphenylphosphonium tetraphenylborate is insoluble in *o*-NPOE. The anion was therefore switched to a comparably hydrophobic oxonol anion [19]. Sensors containing

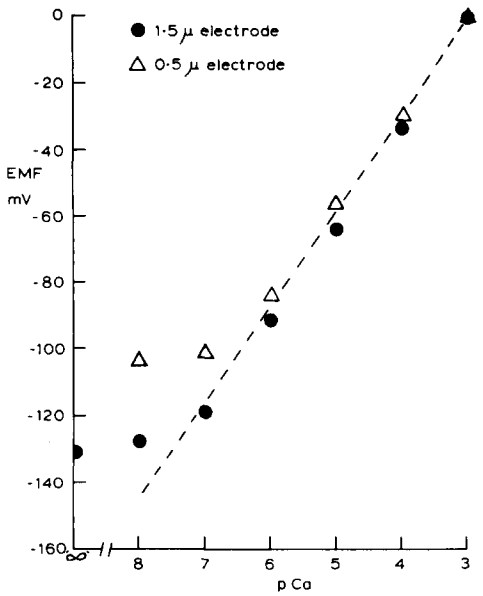

Fig. 2. Calibration plots for two red sensor microelectrodes with tip outer diameters 1.5 and 0.5 μm, as indicated. The dotted line represents the theoretical (Nernstian) response to $[Ca^{2+}]$.

salts of this anion with hydrophilic cations behaved like $NaBPh_4$ sensor, indicating that any changes in electrode performance were not due to the anion substitution. Tetraphenylphosphonium oxonol electrodes hardly ever show calibration slopes significantly greater than Nernstian or hysteresis of more than 2 mV between ascending and descending calibrations in the region $pCa < 7$. A minor additional advantage lies in the brilliant red color of the oxonol, which makes the electrode tip easy to see. One drawback seems to be some reduction of response, particularly between pCa 7 and 8, between which values no more than 11—15 mV can be produced (Fig. 2). Also shown in Fig. 2 is the usual effect of tip diameter, the 0.5 μm electrode having distinctly poorer performance than the 1.5 μm electrode. Decreasing selectivity with decreasing tip size seems to be a general phenomenon in liquid sensor microelectrodes [7,10]. Presumably the decreasing ratios of sensor cross-sectional area to wall thickness and to lumen circumference increase the degree of shunting of the active interface by leakage through the glass wall or along its inner surface.

The smallest electrodes we could reliably make were approx. 0.4 μm tip outer diameter. Smaller electrodes were difficult to fill by the methods described here, and had poor response below 1 μM Ca^{2+}. However, a well beveled 0.5 μm electrode should be able to penetrate most types of cell currently studied by electrophysiological techniques.

Another substitute used for $NaBPh_4$ has been tetrabutylammonium tetraphenylborate. This salt gives some electrode slopes greater than Nernstian, but hysteresis has not been a problem, and a rather better response between pCa 6 and 8 is sometimes obtainable.

Electrical properties of the electrodes

Electrodes with 1.5 μm tip outer diameter have a resistance of 20—30 GΩ in p*Ca* 3 solution; 0.5 μm tips give 60—120 GΩ. The resistance is not noticeably dependent on the sensor column length, which is always greater than 100 μm. With sensor containing $NaBPh_4$, the resistance is not significantly affected by changing the bathing solution. Red sensor gives electrodes which in p*Ca* 3 solution have similar resistances to $NaBPh_4$ sensor, but in p$Ca \geqslant 7$ solution the resistance often increases by 30—50%. A large and changing electrode resistance requires the electrometer to have a very high input impedance and a small and stable bias current (less than 10 fA) if spurious signals are to be avoided. We have found it necessary to recheck bias current and input impedance frequently and not to rely on manufacturers' claims, since slight temperature changes, stray leakages, and subtle component failures have all been seen to worsen bias currents and input impedances to far beyond specifications.

Interference from other ions

We consider that the most severe test for interference from ions other than Ca^{2+} is to record the response to changes in their concentration in solutions with virtually zero [free Ca^{2+}], i.e., 10 mM EGTA and no added Ca^{2+}. This approach has the additional advantage that one need not know in detail the effect of the competing ion on the buffer ligand. For example, Na^+ binds more strongly than does K^+ to EDTA [15] and probably to EGTA [20]. Thus simple replacement of K^+ by Na^+ in, say, the p*Ca* 7 formula of Table I could affect the [EGTA] to increase free [Ca^{2+}] and cause a rise in e.m.f. difficult to separate from the true interference of Na^+ on the electrode. If however there is practically zero total Ca^{2+}, the free Ca^{2+} cannot rise significantly.

H^+. In many tests with all sensor varieties, the e.m.f. in p*Ca* '∞' solutions never increased by more than 4 mV in going from pH 8.5 to 6.5, and usually by only 1—2 mV. At higher [Ca^{2+}], e.g., p*Ca* 7 and 6, no response to pH shifts between 7.7 and 6.8 could be seen (p*Ca* maintained constant by change of Ca^{2+} buffer).

Mg^{2+}. In our hands the neutral ligand sensor is remarkably selective for Ca^{2+} over Mg^{2+}. Typically the e.m.f. rises by only 1 mV when the Mg^{2+} concentration is raised from zero (no Mg^{2+} added) to 20 mM free (25 mM total $MgCl_2$, 7 mM EGTA at pH 7.50 in 100 mM KCl). Some electrodes have given slightly greater responses to Mg^{2+} — at most 2—3 mV going from 0 to 5 mM free Mg^{2+} in p*Ca* '∞' solutions.

K^+. The limit to the response to low [Ca^{2+}] in these calibration solutions is imposed by interference from the approx. 125 mM K^+. Replacement of K^+ by choline in p*Ca* '∞' results in a potential 30—40 mV more negative. However, alterations of KCl concentration in the range 85—175 mM with p*Ca* '∞' produce responses of only 4—5 mV. Alterations of K^+ concentration likely to be encountered inside intact cells should therefore not cause significant e.m.f. fluctuations.

Na^+. Sodium causes significant interference. Isotonic substitution of 20 mM Na^+ for K^+ in p*Ca* '∞' usually gives an approx. 4 mV rise in e.m.f. Complete replacement of the 125 mM K^+ by Na^+ gives 20—25 mV. Fortunately, the free Na^+ concentration inside vertebrate cells rarely reaches 20 mM; if it does, it will

give rise at low $[Ca^{2+}]$ to a significant error unless the appropriate correction is made. At higher $[Ca^{2+}]$, p$Ca < 6$, the replacement of 20—30 mM K^+ by Na^+ usually has less than 1 mV effect.

The preceding discussion has avoided citing selectivity coefficients for Ca^{2+} vs. competing cations, for the following reasons. (a) At least three equations are available [21] for the e.m.f. as a function just of the activities of Ca^{2+} and one monovalent interfering ion, M^+:

$$\text{e.m.f.} = \text{constant} + (RT/F)\ln(a_{Ca}^{1/2} + K_1 a_M) \quad (1)$$

$$\text{e.m.f.} = \text{constant} + (RT/2F)\ln(a_{Ca} + K_2 a_M^2) \quad (2)$$

$$\text{e.m.f.} = \text{constant} + (RT/F)\ln[(a_{Ca} + 0.25\,K_3 a_M^2)^{1/2} + 0.5\,K_3^{1/2} a_M] \quad (3)$$

Since these equations are mathematically nonequivalent, no more than one of them can be right, and the value quoted for the selectivity constant (K_1, K_2 or K_3) depends strongly on the choice. (b) Whichever equation is chosen, the estimate for the selectivity coefficient depends markedly on the solutions used for calibration. For example, separate solutions of pure salts lead to values different [18,22] from those obtained with solutions such as those of Table I, where a fixed concentration of interfering ions (K^+) is mixed with varying levels of the primary ion, Ca^{2+}. Even if the mixed solution method is accepted, the apparent selectivity coefficient will vary with the K^+ concentration, since Eqns. 1—3 do not correctly describe the dependence of e.m.f. on the K^+ activity when Ca^{2+}, Mg^{2+} and Na^+ are absent. Since selectivity coefficients for these electrodes are so dependent on theoretical assumptions and experimental details, it seems simpler just to report the observed effects of interfering ions.

Speed of response. Even with electrodes selected for good calibration slope, the response time has been rather variable. The response to changes in the range p*Ca* 3—5 is almost always complete within the fraction of a second taken to flush the new solution through. Other workers have also reported very fast responses at high $[Ca^{2+}]$ levels [23]. However, the time resolution becomes progressively worse at lower p*Ca* and typically many seconds are required for a 95% complete response going from p*Ca* 5 to 6 or from p*Ca* 6 to 7. This time course is typically nonexponential and as much as two orders of magnitude slower than can be explained by the product of electrode resistance and total input capacitance. Of the electrodes checked for speed, the fastest produced a virtually complete response from p*Ca* 7 to 6 in 3 s and 6 to 5 within 1 s. The fastest responses are usually obtained with beveled electrodes, but not all beveled electrodes are fast.

Stability. Two main forms of instability have been observed: drift, i.e., a shift of the entire calibration curve along the e.m.f. axis; and loss of slope, i.e., a decrease in Δe.m.f./Δp*Ca* at the lowest $[Ca^{2+}]$ levels. Electrodes commonly show most drift, say 1—2 mV/min, shortly after fabrication and during the first part of the calibration procedure. After 10—30 min the drift usually becomes smaller. Electrodes which persist in drifting more than approx. 1 mV/min are not normally used for experiments. In practice, the drift of electrodes used inside cells is less than 0.5 mV/min, often less than 0.1 mV/min, as judged by the e.m.f. of the bathing solution before impalement and after withdrawal. The

observed drifts during calibration are approximately linear in time, not stepwise, so the reference e.m.f. for intracellular experiments can be taken from the linear interpolation of the pre- and post-impalement values. A few electrodes have remained stable within a millivolt or so over two days. Obviously drift-free electrodes are desirable, but baseline shifts typically of less than 0.5% full scale response per min are not necessarily unusable in practice. Red sensor seems to make electrodes with less drift than $NaBPh_4$ sensor.

Loss of slope is a progressive flattening of the calibration curve specifically at low $[Ca^{2+}]$ levels. In our hands, all electrodes suffer some loss of slope as they age (even poly(vinyl chloride) macroelectrodes to some extent), but the rate of deterioration is highly variable and not obviously correlated with overall drift of e.m.f. Electrodes for intracellular use should be calibrated and used as soon as possible after they are made. We have investigated a number of mechanisms for loss of slope and attempted to block them.

(a) The hydrophobic silicone coating on the glass could be failing. Creep of the aqueous phase along the walls would obviously be catastrophic if the internal and external phases were bridged. However, the electrode resistance in fact usually increases with aging. Also, siliconized electrode tubing shows no significant change in contact angle between glass and aqueous solutions over prolonged periods.

(b) The glass could become hydrated, allowing current to flow through the walls (as distinct from the previous hypothesis in which current flowed along the surface of the walls). Such hydration has been blamed for tip potentials of ultrafine open-tipped microelectrodes [24], and there is some evidence that even well-bonded silicone coatings hinder only slightly the hydration of underlying silica [25]. Against this hypothesis is the progressive increase in electrode resistance cited above, plus three other observations: the pH sensitivity of the aging electrode usually does not increase; storing electrodes in air instead of in solution does not stop the deterioration; aluminosilicate glass, known to have much greater electrical resistivity and resistance to hydration than borosilicate [5], has not produced better electrodes.

The remaining hypotheses postulate changes in the liquid sensor at the tip rather than in the insulation. Such hypotheses are favored by the observation that electrodes losing calibration slope can often rejuvenated by expelling a tiny drop of sensor out of the tip or by sucking fresh sensor in.

(c) The hydrophobic anion (tetraphenylborate or oxonol) could be leaching into the bathing solutions. Loss of the ionic salt would raise the resistance of the electrode, as observed, and decrease the selectivity at low $[Ca^{2+}]$. (Sensors made up without any salt have little response below p*Ca* 5.) Tetraphenylborate can enter and leave the organic phase under some circumstances, despite theoretical assumptions [26] that it cannot. Thus tetraphenylborate in sub-millimolar concentrations in the bathing solutions pushes the e.m.f. as much as several hundred millivolts more negative. This e.m.f. shift is largely (albeit only slowly) reversible on removal of the tetraphenylborate. Also $NaBPh_4$ at 0.6 mM in *o*-NPOE is in equilibrium with 0.1 mM in 0.1 M NaCl. Unfortunately, attempts to prevent or reverse anion loss failed to increase electrode longevity. Thus $NaBPh_4$ was replaced by sodium tetrakis(*p*-biphenylyl)borate [27], in which an extra phenyl group is attached to each of the four phenyl groups of

tetraphenylborate. The partition coefficient into the organic phase should thereby increase by some eight orders of magnitude [28]. But the electrodes showed no improvement. Alternatively, deteriorated $NaBPh_4$ electrodes were soaked in aqueous solutions of $NaBPh_4$, sometimes with bias currents to help pull the anion into the sensor. Such conditioning caused large e.m.f. drifts during subsequent recalibration, but little or no restoration of the lost response to low $[Ca^{2+}]$ levels.

(d) Our earliest method of siliconization was the well-known method of exposure of undried glass to dimethyldichlorosilane. This procedure is believed to form a film (tens or hundreds of nanometers thick) of poly(dimethylsiloxane), much of which is not chemically bonded to the glass [29]. If this silicone oil layer were to dissolve in the sensor, it would depress the dielectric constant, a change which is known to harm the selectivity for divalent over monovalent cations [30]. Indeed, sensor pre-equilibrated with the hydrolysis product of dimethyldichlorosilane makes electrodes which from the moment of filling give calibration plots looking like those of severely deteriorated normal electrodes. The treatment with tributylchlorosilane vapour described in Methods was designed to remove adsorbed water before forming a covalently bonded, strongly hydrophobic layer one molecule thick. Unlike dimethyldichlorosilane, the tributylchlorosilane once bonded cannot retain a chlorine atom which would hydrolyze to a hydrophilic OH group on later exposure to moisture.

(e) Initially the electrolyte inside the electrode was 0.1 M or 1 M $CaCl_2$ [1,9]. Surprisingly, the use of a buffered low $[Ca^{2+}]$ electrolyte (p*Ca* 7) substantially slowed down the deterioration in calibration slope. The explanation of this empirical finding is not clear. Certainly, too much Ca^{2+} in the sensor (for example a 1 : 1 mole ratio of $Ca(BPh_4)_2$ to neutral ligand) completely destroys the response to aqueous Ca^{2+}. But it is not obvious that Ca^{2+} in the internal electrolyte could reach the sensor or at the tip within minutes after filling. Perhaps the initial filling with $CaCl_2$ deposits some Ca^{2+} which is not displaced by the inflow of sensor but later dissolves in the sensor. Other [free Ca^{2+}] levels for the internal electrolyte have not been tested. Reduction of the K^+ content to less than 10 mM gave no advantage and made it more difficult to monitor electrode resistance during beveling.

Conclusions

The performance of these Ca^{2+}-selective microelectrodes indicates that they should be able to monitor [free Ca^{2+}] levels inside cells. The smallest electrodes have only just adequate performance and further improvements in electrode selectivity, speed, and stability are certainly desirable. Even if such technical improvements are achieved, two more fundamental problems will always have to be considered. The first is that a microelectrode takes a point sample of the $[Ca^{2+}]$ level and can easily miss a localized change in [free Ca^{2+}] which could be functionally very important. A likely example would be sub-membrane $[Ca^{2+}]$ transients associated with inward Ca^{2+} currents, e.g. in secretory cells. The second caveat is that unidentified constituents of the cytoplasm could perhaps be deranging the electrode. We already know of two nonphysiological

substances, tetraphenylborate and silicone oil, which can greatly alter electrode characteristics. One strategem to assess interference would be to buffer samples of cytoplasm to precisely known [Ca^{2+}] levels and see if the electrode reads properly. Unfortunately, in most tissues this would be technically rather difficult. Another approach would be to compare electrode results with [Ca^{2+}] estimates obtained by independent techniques the possible sources of error of which are mostly different from those affecting electrodes. So far the results with electrodes give values encouragingly similar to determinations by aequorin luminescence in the two tissues in which data for comparison have been available, barnacle giant muscle fibers [9] and frog sartorius [31]. Fig. 3 shows one record obtained from a frog sartorius fiber. The e.m.f. recorded here indicated a sarcoplasmic p*Ca* of approx. 6.8 (0.16 μM). Some other impalements gave similar [Ca^{2+}] levels, but in other cells levels of up to 10 μM were recorded. In many fibers, including that shown in Fig. 3, the intracellular voltage electrode recorded a marked depolarization on insertion of the Ca^{2+} electrode, presumably reflecting penetration damage which could also elevate the local [Ca^{2+}].

In trabeculae from ferret ventricular myocardium, a quite wide range of resting [Ca^{2+}] levels, 100 nM to 10 μM, was recorded, the higher levels again possibly due to penetration damage of the very small cells (12—15 μm diameter). Since damage is likely to raise intracellular [Ca^{2+}], the lowest values are presumably the most realistic. Quite substantial increases, e.g., 1—10 μM, were seen during contractures produced by exposure to solutions of caffeine in low [Na^+] (Marban, E., Rink, T.J., Tsien, R.Y. and Tsien, R.W., unpublished data).

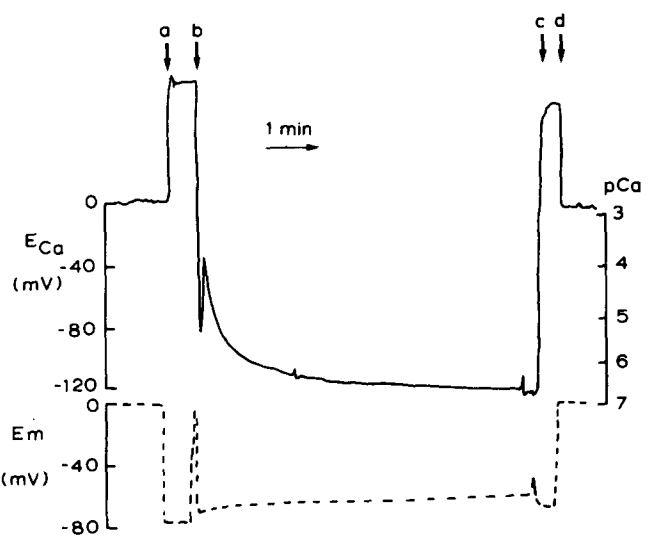

Fig. 3. Measurement of sarcoplasmic [free Ca^{2+}] in a resting frog sartorius muscle fiber, using a beveled 1 μm electrode. E_m is the membrane potential recorded with a standard 20 MΩ microelectrode filled with 3 M KCl. E_{Ca} is the potential between the Ca^{2+}-selective electrode and the KCl-filled electrode. At *a*, the fiber was impaled with the KCl-filled electrode and at *b* with the Ca^{2+} electrode, about 150 μm distant; *c* and *d* indicate withdrawal of the electrodes in reverse order. The right-hand ordinate gives the calibration of the Ca^{2+} electrode. Correspondence between calibration e.m.f. and intracellular E_{Ca} values was established by measuring each with respect to the Ringer's solution containing 1.5 mM $CaCl_2$.

In *Xenopus laevis* embryonic cells, the mean resting *pCa* was 6.5 ± 0.12 (S.E., $n = 12$). [Free Ca^{2+}] was monitored during cell divisions and CO_2-induced electrical uncoupling of the cells [32].

Finally, it seems worth outlining some of the relative advantages and disadvantages of Ca^{2+}-selective microelectrodes vs. optical techniques using photoproteins or dyes. The optical methods have somewhat better Na^+ rejection, no vulnerability to artifacts due to liquid junction potentials or incorrect subtraction of membrane potential; faster responses; and the ability to give either an estimate of [Ca^{2+}] levels averaged over the entire area of observation, or in favorable preparations an indication of the spatial distribution of [Ca^{2+}] levels [33]. Electrodes are superior in the following aspects: Mg^{2+} and H^+ rejection; very wide range of [Ca^{2+}] levels quantitatively measurable; relative ease of incorporation into existing electrophysiological arrangements; lack of artifacts due to incomplete diffusion of an indicator; indifference to native pigments or light scattering in the specimen. Elucidation of the role of Ca^{2+} inside cells will require all available monitoring techniques, and it is hoped that the neutral-carrier microelectrodes will have their own important part to play.

Appendix

In this appendix we try to explain something of what happens inside a Ca^{2+} electrode within the first few minutes after a change in the external solution, to account for the following observations:

(1) A certain concentration of an anion, on the order of 10^{-2} M, must be present in the sensor for good selectivity, but too much $NaBPh_4$ or $Ca(BPh_4)_2$ (approx. 10^{-1} M) ruins the selectivity.

(2) Na^+ salts or Ca^{2+} salts are more prone than salts of large hydrophobic cations to give electrodes with hysteresis, greater-than-Nernstian calibration slopes, and slow drooping responses at low [Ca^{2+}] levels.

(3) Na^+ salts or Ca^{2+} salts make electrodes the resistance of which is practically independent of the bathing [Ca^{2+}] level, whereas electrodes with tetraphenylphosphonium increase significantly in resistance as the [Ca^{2+}] level falls.

Morf, Simon, and coworkers [21,26,34] have presented formal mathematical models of neutral carrier membrane electrodes. Under certain assumptions these models predict steady-state e.m.f. values to obey the Nicolsky equation or a close analogue. However, microelectrodes with sensor column lengths of several hundred μm would take at least tens of minutes or hours to reach the assumed steady state throughout the column. Morf et al. [35] have also demonstrated that $NaBPh_4$ in the sensor inhibits interference from anions such as thiocyanate or perchlorate which would otherwise depress the e.m.f. at high [Ca^{2+}]. The effect of $NaBPh_4$ to improve selectivity for divalent over monovalent cations was noted but not discussed.

Our explanation for the effect of lipophilic anions in boosting Ca^{2+}: M^+ selectivity is that there is some formation of ion-pairs [36] between the anions and the metal-ligand complexes. Such ion-pairing should be more exergonic for doubly-charged Ca complexes than for singly-charged Na or K complexes. The transfer of Ca^{2+} from the aqueous phase into the sensor should thus be facilitated to a greater extent than the transfer of monovalant cations. Adding

anions should and does have qualitatively the same effect as raising the dielectric constant of the solvent [30].

Too much Na^+ or Ca^{2+} ruins the selectivity because the cations fill all the available ligand sites, preventing the entry of fresh Ca^{2+} from the aqueous phase. K^+ dominates the potential because it is most able to enter the organic phase without the help of the ionophore, just as in the common K^+ liquid ion exchanger (Corning 477317) which is simply an organic solution of potassium tetrakis(*p*-chlorophenyl)borate [14].

The explanation of hysteresis and droop is simplest if the sensor be made up with a uniform concentration of $Ca(BPh_4)_2$. On exposure to a high concentration of Ca^{2+} (say, p*Ca* 3 in 100 mM KCl), an efflux of Ca^{2+} from the sensor must be balanced by an influx of cations from the aqueous solution, since the net electrical current is zero. This influx must be practically all Ca^{2+}, since the electrode gives a Nernstian response to $[Ca^{2+}]$ at such high levels. Imagine now instantaneously changing the solution to a very low concentration of Ca^{2+}, say p*Ca* 8. For a few seconds the Ca^{2+} efflux is not balanced by the cation influx; the net current serves to charge the circuit capacitance as the e.m.f. shifts in a negative direction. The change in potential across the sensor/water interface retards the Ca^{2+} efflux and encourages cation influx, which now has to be carried largely by K^+, since Ca^{2+} is so scarce in the aqueous phase. In a few seconds or less, the Ca^{2+} efflux and K^+ influx come into balance and the fast phase of the e.m.f. response ends. However, the electroneutral Ca^{2+}/K^+ exchange continues. The Ca^{2+} entering the aqueous phase is simply sequestered by the large excess of EGTA buffer. But in the tip of the electrode, K^+ gradually replaces Ca^{2+} over many minutes. Now the ionic selectivity of the electrode suggests that the ionophore prefers Ca^{2+} over Na^+ over K^+. Therefore K^+ in the sensor is likely to have a higher propensity than Ca^{2+} (or Na^+) to escape back into the aqueous phase. As K^+ replaces Ca^{2+}, the sensor potential must become more negative to maintain zero electrical current. This is the proposed explanation for the slow e.m.f. droop on standing in low $[Ca^{2+}]$ solutions. If the $[Ca^{2+}]$ level of the aqueous solution is now raised, the electrode potential rises somewhat, but until the K^+ in the tip is fully replaced again by Ca^{2+}, the potential remains less than the value recorded approaching from higher $[Ca^{2+}]$ levels. If this restoration takes too long, the electrode effectively shows hysteresis.

The above argument should hold also for a $NaBPh_4$-filled sensor, since exposure to high $[Ca^{2+}]$ should cause replacement of the Na^+ in the tip by Ca^{2+}. Even if this exchange is not yet complete when the bathing solution is changed to a low $[Ca^{2+}]$ level, the probable preference of the ionophore for Na^+ over K^+ would give Na^+/K^+ exchange the same qualitative effect as Ca^{2+}/K^+.

Our main interest in electrodes was in their use as biological tools rather than in their physical chemistry, so our test of this mechanism has been to try to block electroneutral ion-exchange by replacing exchangeable cations by intrinsically hydrophobic cations which should be practically unable to leave the organic phase. With the hydrophobic cations and anions in stoichiometric balance, electroneutrality of the bulk interior of the sensor requires that metal-ligand complexes stay in surface charge layers close to and in rapid equilibrium with the aqueous phase. This cation substitution did have approximately the

predicted and desired effect (see Results above), but the possibility remains that the successful cure was based on a fallacious diagnosis.

The above model of an Na^+- or Ca^{2+}-loaded sensor postulates reasonably large cation fluxes in and out of the sensor, whatever the $[Ca^{2+}]$ level. Therefore the electrical resistance of the interface is likely to be small and not too greatly dependent on p*Ca*. The observed overall resistance of the electrode, including the series resistance of the bulk sensor, should be nearly constant. A tetraphenylphosphonium-loaded sensor should have much smaller resting fluxes especially at low $[Ca^{2+}]$ levels, so the overall resistance has a larger component sensitive to external p*Ca*.

One might ask whether the tetraphenylphosphonium cations give the electrode some anion-selective properties. After all, most anion-selective liquid ion exchangers are just solutions of salts of quaternary hydrophobic cations. Anion selectivity would show itself as a flattening of the response slope at high $[Ca^{2+}]$, where the highly positive e.m.f. values would most encourage the anions to enter the sensor. In fact, no such flattening is observed up to at least p*Ca* 3. Moreover, the original $NaBPh_4$ formulation contained large cations, also — the ligand complexes of Na^+, or Ca^{2+} or K^+ depending on the history of bathing solutions.

Acknowledgements

This work was supported by grants from the Science Research Council. We thank Professor W. Simon for gifts of sensor components, Ms. K. Clarke for collaboration in some of the frog muscle experiments, Mr. A. Dunn for technical assistance, Dr. M. Berridge for helpful discussion, and Dr. F.J. Alvarez-Leefmans for comments on the manuscript.

References

1 Oehme, M., Kessler, M. and Simon, W. (1976) Chimia (Aarau) 30, 204—206
2 Duncan, C. (ed.) (1976) Calcium in Biological Systems, Symp. Soc. Exptl. Biol., Vol. 30
3 Scarpa, A. and Carafoli, E. (eds.) (1978) Calcium Transport and Cell Function, Ann. N.Y. Acad. Sci., Vol. 307
4 Ashley, C.C. and Campbell, A.K. (eds.) (1979) Detection and Measurement of Free Calcium Ions in Cells, Elsevier, Amsterdam
5 Thomas, R.C. (1978) Ion-sensitive Intracellular Microelectrodes, Academic Press, New York
6 Christoffersen, G.R.J. and Simonsen, L. (1977) Acta Physiol. Scand. 101, 492—494
7 Brown, H.M., Pemberton, J.P. and Owen, J.D. (1976) Anal. Chim. Acta 85, 261—276
8 Owen, J.D., Brown, H.M. and Pemberton, J.P. (1977) Anal. Chim. Acta 90, 241—244
9 Ashley, C.C., Rink, T.J. and Tsien, R.Y. (1978) J. Physiol. (Lond.) 280, 27P
10 Sokol, J.H., Lee, C.O. and Lupo, F.J. (1979) Biophys. J. 25, 143a
11 Ammann, D., Bissig, R., Gueggi, M., Pretsch, E., Simon, W., Borowitz, I.J. and Weiss, L. (1975) Helv. Chim. Acta 58, 1535—1548
12 Oehlschlaeger, H., Riester, O., Mueller, H. and Reckziegel, E. (1971) British Patent 1, 231, 884
13 Latimer, P. (1979) Biophys. J. 27, 117—126
14 Coles, J.A. and Tsacopoulos, M. (1979) J. Physiol. (Lond.) 290, 525—549
15 Martell, A.E. and Smith, R.M. (1974) Critical Stability Constants, Vol. 1, Plenum Press, New York
16 Schwarzenbach, G., Senn, H. and Anderegg, G. (1957) Helv. Chim. Acta 40, 1886—1900
17 Boyd, S., Bryson, A., Nancollas, G.H. and Torrance, K. (1965) J. Chem. Soc. 7353—7358
18 Moody, G.J. and Thomas, J.D.R. (1971) Selective Ion-Sensitive Electrodes, pp. 4—24, Merrow Publishing Co., Watford, U.K.
19 Tsien, R.Y. (1976) Ph.D. Thesis, University of Cambridge

20 Tanaka, M., Funahashi, S. and Shirai, K. (1968) Inorganic Chem. 7, 573—578
21 Morf, W.E., Ammann, D., Pretsch, E. and Simon, W. (1973) Pure Appl. Chem. 36, 421—439
22 Ammann, D., Meier, P.C. and Simon, W. (1979) in Detection and Measurement of Free Calcium Ions in Cells (Ashley, C.C. and Campbell, A.K., eds.), Elsevier, Amsterdam
23 Heinemann, U., Lux, H.D. and Gutnick, M.J. (1977) Exp. Brain Res. 27, 237—243
24 Agin, D.P. (1969) in Glass Microelectrodes (Lavallee, M., Schanne, O.F. and Hebert, N.C., eds.), pp. 62—75, Wiley, New York
25 Stoeber, W. (1956) Kolloid Z. 149, 39—46
26 Morf, W.E., Kahr, G. and Simon, W. (1974) Anal. Lett. 7, 9—22
27 Holzapfel, H. and Richter, C. (1964) J. Prakt. Chem. 26, 15—23
28 Leo, A., Hansch, C. and Elkins, D. (1971) Chem. Rev. 71, 525—616
29 Vanderwort, G.L. and Willard, J.E. (1948) J. Am. Chem. Soc. 70, 3148
30 Fiedler, U. (1977) Analyt. Chim. Acta 89, 111—118
31 Blinks, J.R., Rudel, R. and Taylor, S.R. (1978) J. Physiol. (Lond.) 277, 291—323
32 Rink, T.J., Tsien, R.Y. and Warner, A.E. (1980) Nature 283, 658—660
33 Rose, B. and Loewenstein, W.R. (1975) Nature 254, 250—252
34 Morf, W.E., Wuhrmann, P. and Simon, W. (1976) Anal. Chem. 48, 1031—1039
35 Morf, W.E., Ammann, D. and Simon, W. (1974) Chimia (Aarau) 28, 65—67
36 Buechi, R., Pretsch, E., Morf, W.E. and Simon, W. (1976) Helv. Chim. Acta 59, 2407—2416