Long-term uncoupling of chloride secretion from intracellular calcium levels by Ins(3,4,5,6)P_4

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OSMOREGULATION, inhibitory neurotransmission and pH balance depend on chloride ion (Cl^-) flux. In intestinal epithelial cells, apical Cl^- channels control salt and fluid secretion and are, in turn, regulated by agonists acting through cyclic nucleotides and internal calcium ion concentration ($[Ca^{2+}]_i$)[1–3]. Recently, we found that muscarinic pretreatment prevents $[Ca^{2+}]_i$ increases from eliciting Cl^- secretion in T_{84} colonic epithelial cells[4]. By studying concomitant inositol phosphate metabolism, we have now identified D-*myo*-inositol 3,4,5,6-tetrakisphosphate (Ins(3,4,5,6)P_4), as the inositol phosphate most likely to mediate this uncoupling. A novel, membrane-permeant ester prepared by total synthesis delivers Ins(3,4,5,6)P_4 intracellularly and confirms that this emerging messenger[5] does inhibit Cl^- flux resulting from thapsigargin- or histamine-induced $[Ca^{2+}]_i$ elevations.

In the human colonic epithelial cell line T_{84} (ref. 6), carbachol initally stimulates a transient elevation of $[Ca^{2+}]_i$ and rise in Cl^- secretion which return to near-control levels within 10 min[7,8]. However, prolonged pretreatment with carbachol blocks subsequent thapsigargin-stimulated Cl^- secretion, without altering the ability of thapsigargin to increase $[Ca^{2+}]_i$[4]. This does not appear to be mediated by protein kinase C (PKC)[9]. Similarly, we have now found that pretreatment with carbachol reduced 10^{-4}M histamine-stimulated Cl^- secretion by an average of 77% (peak change in short circuit current (ΔI_{sc}) ($\mu A\ cm^{-2}$); $n=3$, $P<0.026$) without altering the $[Ca^{2+}]_i$ response to histamine (ref. 8, and data not shown). Therefore, carbachol generates an intracellular signal capable of uncoupling the Cl^- secretory response from $[Ca^{2+}]_i$. We investigated the time course of this uncoupling. Carbachol-mediated inhibition of thapsigargin-stimulated Cl^- secretion was evident within 11 min, maximal by 20 min, and was maintained for more than 90 min (Figs 1*a* and 2*a*). The persistence of this phenomenon suggests the long-term effect of muscarinic agonists on Cl^- secretion will be inhibitory.

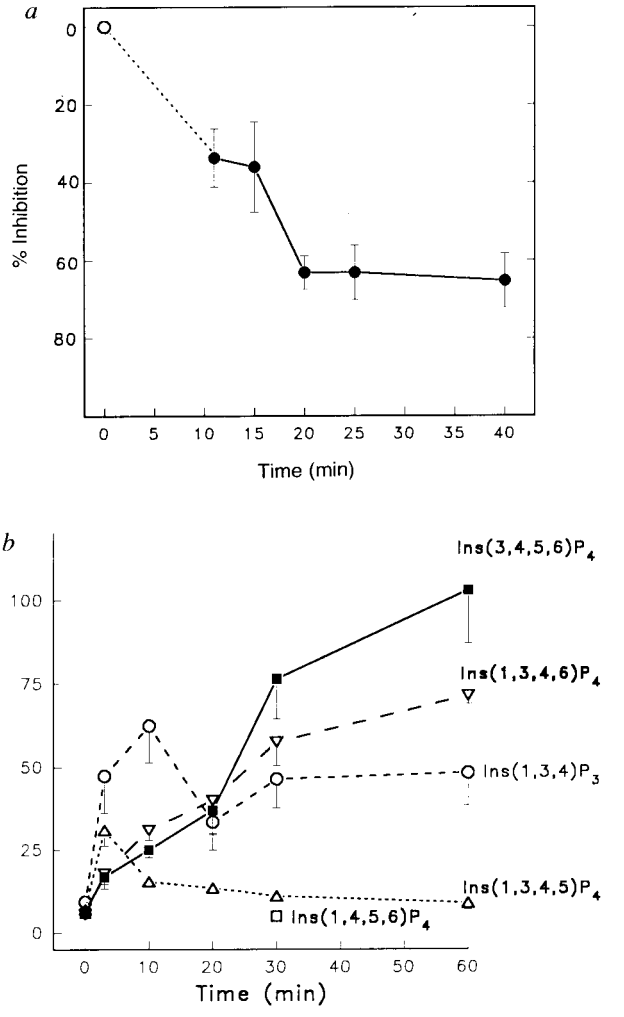

FIG. 1 Comparison of the time course of the inhibition of Ca^{2+}-stimulated Cl secretion and carbachol-induced elevation of inositol phosphates in T_{84} cells. *a*, Carbachol-mediated inhibition of thapsigargin-stimulated Cl secretion relative to the 10 min, peak response to thapsigargin alone (open circle) is depicted. Carbachol (10^{-4} M) was added to T_{84} cell monolayers mounted in Ussing chambers and, at subsequent intervals up to 30 min, thapsigargin (Tg, 10^{-6} M) was added. After a further 10 min Cl secretion was assayed as the change in short circuit current (ΔI_{sc} $\mu A\ cm^{-2}$) as described[7]. Data are means ± s.e.m. for $n=5$. *b*, Carbachol (10^{-4} M) was added to cells prelabelled with [^{3}H]inositol for 72 h and levels of inositol phosphates were measured at the indicated intervals. Data are c.p.m. expressed as a fraction of total cellular radioactivity and are means ± s.e.m., $n=4$. The techniques used to label the cells with [^{3}H]inositol, and the methods for quenching the cells, extracting the inositol phosphates, and resolving the isomers on Adsorbosphere SAX HPLC columns, are all as described previously[4]. [^{3}H]Ins(3,4,5,6)P_4 and [^{3}H]Ins(1,4,5,6)P_4 are enantiomers and elute from the column as a single peak. Their relative amounts were determined by incubating the peak with ^{33}P-Ins(1,4,5,6)P_4 and partially purified Ins(1,4,5,6)P_4 3-kinase[13]. A comparison of zero-time peaks (56% ± 8% [^{3}H]Ins(1,4,5,6)P_4, 44 ± 8% [^{3}H]Ins(3,4,5,6)P_4, $n=5$) with peaks that increased sevenfold after 30 min of stimulation with carbachol (5–7% [^{3}H]Ins(1,4,5,6)P_4, 93–95% [^{3}H]Ins(3,4,5,6)P_4, $n=2$) indicated that carbachol specifically increased levels of [^{3}H]Ins(3,4,5,6)P_4 roughly 15-fold whereas levels of [^{3}H]Ins(1,4,5,6)P_4 were unchanged. Similar changes were observed when Ins(3,4,5,6)P_4 mass was measured directly (Fig. 4). Thus, levels of [^{3}H]Ins(3,4,5,6)P_4 throughout the time course were calculated by substracting the amount of [^{3}H]Ins(1,4,5,6)P_4 at zero time from the other time points.

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Carbachol stimulates the formation of many inositol polyphosphates, any of which might be the uncoupling signal. Levels of a plausible candidate should rise and fall with a time course matching that of the inhibition of Ca^{2+}-dependent Cl^- secretion. The transient increases in $[^3H]Ins(1,4,5)P_3$ and $[^3H]Ins(1,3,4,5)P_4$ returned to baseline within 3 min[4] and 20 min (Fig. 1*b*), respectively, even while inhibition of Cl^- secretion was maximal (Fig. 1*a*). Levels of $[^3H]Ins(1,4,5,6)P_4$ (Fig. 1*b*), $[^3H]Ins(1,3,4,5,6)P_5$ (ref. 4) and $[^3H]InsP_6$ (ref. 4) were not elevated after carbachol stimulation. Reversal of carbachol-mediated inhibition of thapsigargin-stimulated Cl^- secretion was delayed when prolonged carbachol stimulation preceded the addition of atropine (Fig. 2*a*). Thus, because levels of $[^3H]Ins(1,3,4,6)P_4$ and $[^3H]Ins(3,4,5,6)P_4$ and $[^3H]Ins(1,3,4)P_3$ (which were all elevated at later time points corresponding to inhibition of Cl^- secretion, Fig. 1*b*) returned to baseline at different rates following addition of atropine (Fig. 2*b*), it was possible to narrow the correlation further. $Ins(3,4,5,6)P_4$ was the only inositol phosphate to remain elevated above basal levels while Cl^- secretion remained inhibited (Fig. 2). However, the

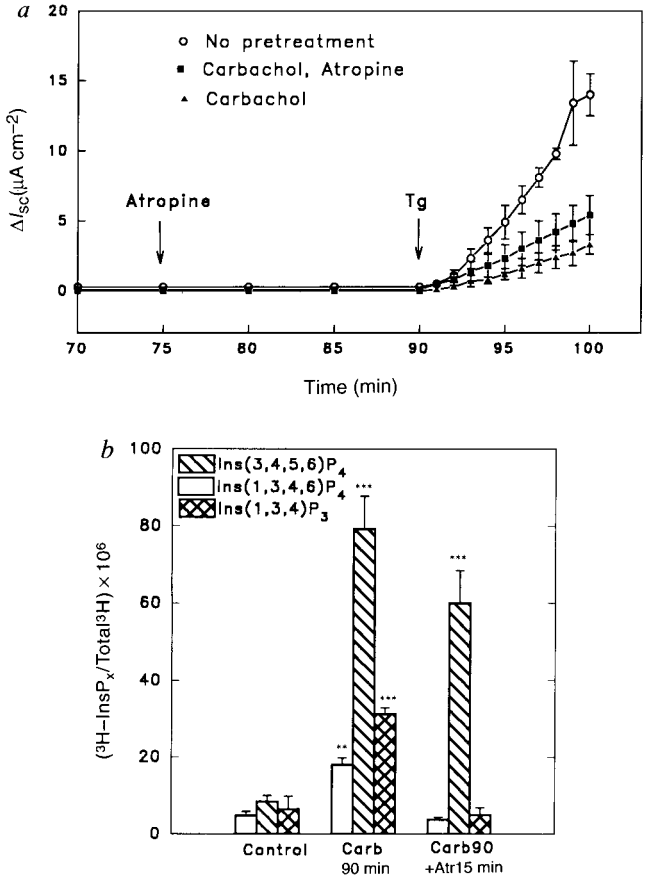

FIG. 2 Effect of atropine on carbachol-mediated increases in inositol phosphates and inhibition of Ca^{2+}-dependent Cl^- secretion. *a*, Time course of thapsigargin (Tg)-stimulated Cl^- secretion with no pretreatment or after pretreatment with 10^{-4} M carbachol (added at time 0) with or without the addition of atropine (10^{-6} M) 15 min before Tg addition. Cl secretion across T_{84} monolayers mounted in Ussing chambers was monitored as short circuit current (I_{sc}) as in Fig. 1. Data are means ± s.e.m. of I_{sc} from individual monolayers, $n=5$. Monolayers were stimulated with carbachol for 90 min or carbachol for 75 min followed by atropine + carbachol for the remaining 15 min before the addition of Tg. Tg alone is shown as 'control'. *b*, Cells were labelled with ^3H-inositol as described[4] and stimulated with carbachol (10^{-4} M) for 90 min. Atropine (10^{-6} M), where present, was added 75 min after carbachol. After an additional 15 min, cells were quenched and inositol phosphates were extracted and analysed by HPLC[4]. Data are c.p.m. expressed as fraction of total cellular radioactivity and are means ± s.e.m., $n=6$. The graph depicts levels of $Ins(3,4,5,6)P_4$, $Ins(1,3,4,6)P_4$ and $Ins(1,3,4)P_3$ under the conditions indicated. All other inositol phosphates were at basal levels after 90 min carbachol + 15 min atropine. (Values that differ significantly from control values are denoted by asterisks: $^{**}P<0.01$; $^{***}P<0.001$ by Student's 2-tailed *t*-test).

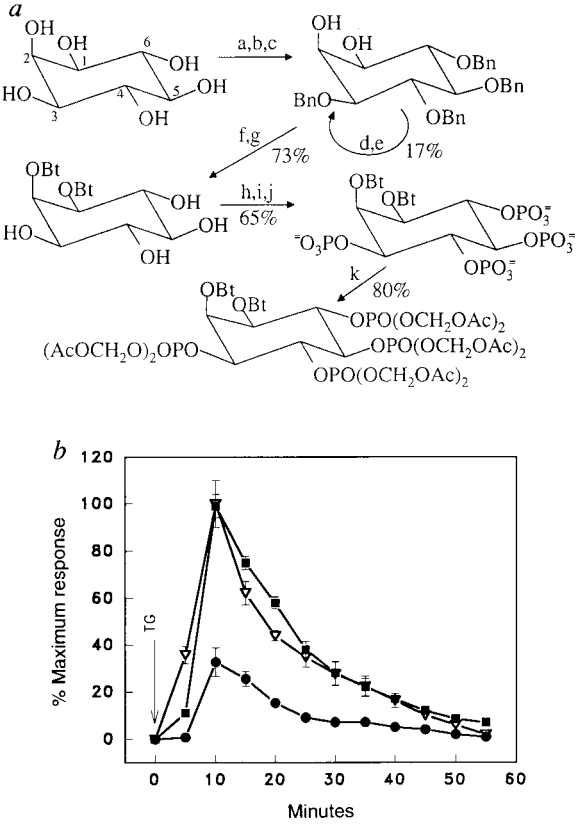

FIG. 3 Synthesis of $Bt_2Ins(3,4,5,6)P_4$/AM and $Bt_2Ins(1,4,5,6)P_4$/AM and effects on thapsigargin-stimulated Cl^- secretion. *a*, Synthesis of $Bt_2Ins(1,4,5,6)P_4$/AM and its racemic mixture with $Bt_2Ins(3,4,5,6)P_4$/AM starting from *myo*-inositol. Bt, *n*-butyryl; Bn, benzyl; Ac, acetyl. Reagents: a, cyclohexanone, H^+; *b*, KOH, BnCl; c, H_3O^+; d, camphanoyl chloride, pyridine, then crystallization and silica gel chromatography to separate the diastereomeric camphanates; e, KOH/MeOH; f, Bt_2O, pyridine; g, H_2/Pd-C; h, $Et_2NP(OBn)_2$, tetrazole; I, CH_3CO_3H; j, H_2/Pd-C; k, *i*-Pr_2NEt, $AcOCH_2Br$. Steps a–c were as described in ref. 15. Yields for each group of subsequent steps are stated below the reaction arrows. The structures are drawn to show the stereochemistry of the active component, $Bt_2Ins(3,4,5,6)P_4$/AM, but the syntheses actually produced either its racemic mixture with the inactive enantiomer, $Bt_2Ins(1,4,5,6)P_4$/AM (when steps d and e were skipped) or the latter alone (in >99% enantiomeric excess verified by chiral HPLC). *b*, T_{84} cell monolayers were treated with 200 μM $Bt_2Ins(3,4,5,6)P_4$/AM + 200 μM $Bt_2Ins(1,4,5,6)P_4$/AM (closed circles) or 400 μM $Bt_2Ins(1,4,5,6)P_4$/AM (closed squares) or buffer (open triangles) for 30 min in Ringers' solution at room temperature before 10^{-6} M Tg-induced Cl^- secretion was measured as ΔI_{sc}. Other control experiments showed that equivalent concentrations of P_i/AM, K^+ butyrate, or Pluoronic/DMSO also were without effect. AM esters were dissolved in Pluronic F127/DMSO, final concentration 0.02%, 0.2%, respectively. Data measured in μA cm^{-2} were normalized to the maximum response obtained in the presence of Tg alone and are means ± s.e.m. ($n=2$–4) from a representative experiment. The average maximum response to Tg in monolayers pretreated with 200 μM $Bt_2Ins(3,4,5,6)P_4$/AM + 200 μM $Bt_2Ins(1,4,5,6)P_4$/AM was 35.2 ± 4.5% of the control, (mean ± s.e.m., 4 experiments in duplicate; $P<0.0001$). Neither $Bt_2Ins(3,4,5,6)P_4$/AM + $Bt_2Ins(1,4,5,6)P_4$/AM nor pure $Bt_2Ins(1,4,5,6)P_4$/AM altered resting levels of $[Ca^{2+}]_i$, or the Tg-mediated increase in $[Ca^{2+}]_i$, as assessed in Fura-2-loaded T_{84} cells grown on cover slips, nor the levels of endogenous inositol phosphates (data not shown).

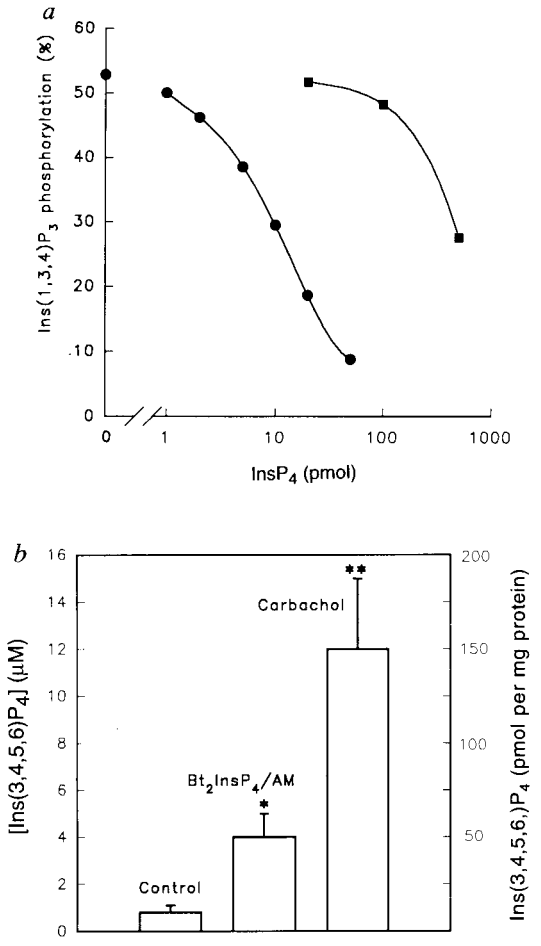

FIG. 4 Determination of levels of Ins(3,4,5,6)P_4 in T_{84} cells treated with vehicle, carbachol or Bt_2Ins(3,4,5,6)P_4/AM. *a*, Standard curves are shown for the inhibition of partially purified Ins(1,3,4)P_3 5/6-kinase[16] by either Ins(3,4,5,6)P_4 or Ins(1,4,5,6)P_4 (kindly supplied by S. Ozaki, Y. Watanabe and M. Hirata, Japan); data are means of duplicates (range <5% of mean) in the same experiment as data shown in *b*. These values were confirmatory of 2 previous experiments. ●, Ins(3,4,5,6)P_4; ■, Ins(1,4,5,6)P_4. Enzyme and inhibitor were incubated with 3 nCi [^{3}H]Ins(1,3,4)P_3 (NEN-DuPont) for 30 min at 37 °C in 250 μl of medium containing 10 mM HEPES (pH 6.6), 6 mM $MgSO_4$, 5 mM ATP, 10 mM creatine phosphate, 1.25 units creatine phosphokinase, 0.08 mg bovine serum albumin. Assays were quenched with perchloric acid, and neutralized with freon/octylamine[17] and analysed by HPLC[4]. Additional, duplicate 5/6-kinase assays contained extracts prepared from T_{84} cells that had been incubated for 30 min with either 0.1 mM carbachol, 200 μM Bt_2Ins(3,4,5,6)P_4/AM + 200 μM Bt_2Ins(1,4,5,6)P_4/AM, or vehicle. These cells were quenched and extracted with phenol/chloroform[18], spiked with 0.4 nCi [^{14}C]Ins(1,3,4,5)P_4 (to monitor the HPLC resolution) + 3 nCi [^{3}H]Ins(1,4,5,6)P_4 (obtained by 3-phosphatase mediated hydrolysis of [^{3}H]Ins(1,3,4,5,6)P_5, 1 Ci mmol^{-1} (ref. 19)). 1-min fractions of HPLC eluates were collected, the Ins(3,4,5,6)P_4/Ins(1,4,5,6)P_4 peak was located and desalted[17] and aliquots were incubated with 5/6-kinase. *b*, The mass of samples were measured as described in *a*. The proportion of Ins(1,3,4)P_3 phosphorylation yielded estimates of the mass levels of Ins(3,4,5,6)P_4. The conversion to concentration was based on a T_{84} cell volume of 10.7 μl per mg protein[20] (control, $n=4$; 200 μM Bt_2(3,4,5,6)P_4/AM + 200 μM Bt_2(1,4,5,6)P_4/AM, $n=3$; 10^{-4} M carbachol, $n=3$; Data are means ± s.e.m.; *$P<0.01$).

inhibition of thapsigargin-induced Cl^- secretion, and the elevation in [^{3}H]Ins(3,4,5,6)P_4 levels by carbachol, were both reversed if a relatively short treatment with agonist (10 min) was accompanied by a prolonged treatment with atropine (50 min); this was the earliest point at which Ca^{2+}-dependent Cl^- secretion was fully restored (106 ± 6% of control values, $n=3$), by which time [^{3}H]Ins(3,4,5,6)P_4 levels had returned to 203 ± 49% of controls. For Ins(3,4,5,6)P_4 to mediate inhibition directly, we would expect its levels to be elevated at least threefold (Fig. 1). An eightfold increase produced full inhibition.

To confirm that Ins(3,4,5,6)P_4 regulates Cl^- transport we directly introduced it into intact cells using cell permeant analogues. Although we were only able to prepare dibutyryl-D-*myo*-inositol(3,4,5,6)P_4/acetoxymethylester (Bt_2Ins(3,4,5,6)P_4/AM) as a racemic mixture that also contained Bt_2Ins(1,4,5,6)P_4/AM, we were able to synthesize enantiomerically pure Bt_2Ins(1,4,5,6)P_4/AM as a control. (See synthesis scheme, Fig. 3*a*). These compounds are cell-permeant because the charged phosphate groups are masked by acetoxymethyl ester (AM) groups[11], whereas the hydroxyl groups are concealed by butyrates. Once inside the cell, the butyryl and acetoxymethyl ester groups are cleaved by intracellular esterases. Thapsigargin-mediated Cl^- secretion was unaffected by 30 min pretreatment of T_{84} cells with 400 μM Bt_2Ins(1,4,5,6)P_4/AM, or vehicle, but was substantially inhibited by 200 μM Bt_2Ins(3,4,5,6)P_4/AM + 200 μM Bt_2Ins(1,4,5,6)P_4/AM (Fig. 3). Thus, the effects are stereospecific for the 3,4,5,6-isomer and are not due to side products of ester hydrolysis. In the continued presence of the Bt_2Ins(3,4,5,6)P_4/AM, inhibition of Cl^- transport lasted at least 45 min. The racemic mixture of 200 μM Bt_2Ins(3,4,5,6)P_4/AM + 200 μM Bt_2Ins(1,4,5,6)P_4/AM also inhibited 10^{-4}M histamine-induced Ca^{2+}-dependent Cl^- secretion by 37.3 ± 4.8% (mean ± s.e.m., $n=3$; $P<0.01$). The critical question of how much intracellular Ins(3,4,5,6)P_4 resulted from extracellular application of the permeant ester could not be answered by measurement of radioactivity, because labelling of the ester prepared as in Fig. 3*a* would require impractical amounts of radioactive inositol. Instead, a novel mass assay for Ins(3,4,5,6)P_4 was devised on the basis of its ability to inhibit Ins(1,3,4)P_3-5/6-kinase *in vitro* (Fig. 4*a*). The results (Fig. 4*b*) depict a 15-fold stimulation by carbachol which agrees with values obtained from radioactive analysis (Fig. 1*b*). The basal level we report here is consistent with previous estimates of [Ins(3,4,5,6)P_4 + Ins(1,4,5,6)P_4] in HL-60 cells[12]. The modest level of Ins(3,4,5,6)P_4 generated from the permeant ester (fivefold over baseline) may reflect some extracellular hydrolysis as well as competition between the 10 steps needed for complete deesterification and the subsequent metabolism of the messenger.

Ins(3,4,5,6)P_4 probably acts without metabolic conversion, because its only significant transformation in T_{84} cells (data not shown) and other tissues[13] is phosphorylation to Ins(1,3,4,5,6)P_5. The latter isomer cannot be responsible because it already exists in unstimulated cells in very high quantities and these levels are relatively static[4]; furthermore, it can also be made from Ins(1,4,5,6)P_4[13], yet Bt_2Ins(1,4,5,6)P_4/AM was biologically inert.

The lack of an exact correlation between the extent and duration of changes in $[Ca^{2+}]_i$ and stimulation of Cl^- secretion in T_{84} cells has been puzzling[8,10]. Here we present a mechanism for this phenomenon by identifying conditions resulting in almost complete dissociation of Cl^- secretion from $[Ca^{2+}]_i$ levels, and by showing that this corresponds to an elevation of Ins(3,4,5,6)P_4 levels. In addition, we have demonstrated a sustained influence of agonists on Cl^- transport long after the origi-

nal stimulus has ceased. This sustained, negative effect contrasts with the more transient and positive signalling responses usually attributed to alterations in inositol phosphate turnover. Furthermore, this effect of Ins(3,4,5,6)P_4 exemplifies a new mechanism for desensitization of phospholipase C-linked responses[14]. Finally, the use of cell-permeant analogues demonstrate a novel means of studying inositol phosphate function in intact cells, which is technically less demanding than microinjecting single cells and is applicable to assays that require the use of cell populations, such as transepithelial secretion. □

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