Pituitary adenyl cyclase-activating peptide (PACAP) stimulates calcium transients and catecholamine secretion in adrenal chromaffin and PC12 cells. The PACAP type 1 receptor in these cells couples to both adenyl cyclase and phospholipase C pathways, but although phospholipase C has been implicated in the response to PACAP, the role of adenyl cyclase is unclear. In this study, we show that PACAP38 stimulates Ca\(^{2+}\) influx in PC12 cells by activating a cation current that depends upon the dual activation of both the PLC and adenyl cyclase signaling pathways but does not involve protein kinase C. In activating the current, PACAP38 has to overcome an inhibitory effect of Ras. Thus, in cells expressing a dominant negative form of Ras (PC12asn17-W7), PACAP38 induced larger, more rapidly activating currents. This effect of Ras could be overridden by intracellular guanosine-5'-O-3-(thio)triphosphate (GTP\(^\gamma\)S), suggesting that it was mediated by inhibition of downstream G proteins. Ras may also inhibit the current through a G protein-independent mechanism, because cAMP analogues activated the current in PC12asn17-W7 cells, provided GTP\(^\gamma\)S was present, but not in PC12 cells expressing wild type Ras. We conclude that coupling of PACAP to both adenyl cyclase and phospholipase C is required to activate Ca\(^{2+}\) influx in PC12 cells and that tonic inhibition by Ras delays and limits the response.

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* The abbreviations used are: PACAP, pituitary adenyl cyclase-activating peptide; PLC, phospholipase C; PKA, cAMP-dependent protein kinase; p75, p75 neurotrophin receptor; pA, pirenzepine; GTP\(^\gamma\)S, guanosine-5'-O-3-(thio)triphosphate; AMP-PCP, adenosine 5'-triphosphate; AMP-PCP, adenosine 5'-triphosphate; GTP\(^\gamma\)S, guanosine-5'-O-3-(thio)triphosphate; db-cAMP, dibutyryl cAMP; 8-Br-cAMP, 8-bromo-cyclic AMP; (R\(_p\))-cAMPS, (R\(_p\))-cyclic adenosine-3',5'-monophosphothioate.

PACAP stimulates calcium transients in a variety of cell types (16–20), including bovine adrenal chromaffin cells (21), by stimulating both the release of Ca\(^{2+}\) from intracellular stores and Ca\(^{2+}\) influx. Some of these responses have been shown to involve PLC activation, but the role of adenyl cyclase is unclear. Since PACAP stimulates inositol 1,4,5-trisphosphate and cyclic AMP production in adrenal chromaffin cells (22), both pathways might be expected to contribute to the response in these cells. We therefore investigated the roles of adenyl cyclase and PLC in the PACAP-induced [Ca\(^{2+}\)]\(_i\) transient in chromaffin-like cells, using the rat PC12 phaeochromocytoma cell line. These cells possess the type I PACAP receptor (23) and, consistent with this, release catecholamines in response to low concentrations of PACAP (24). PACAP also causes PC12 cells to extend neurites and adopt a neuronal morphology that is distinct from that observed for nerve growth factor (23, 25). This effect is independent of the activation of cAMP-dependent protein kinase (PKA), but rather depends on the activation of extracellular signal-regulated kinase 1 or 2 through a Ras-independent mechanism (23). Here we report that, in PC12 cells, PACAP activates a Ca\(^{2+}\)-carrying inward current that is dependent upon the dual activation of both adenyl cyclase and phospholipase C. This current appeared to be negatively regulated by Ras, partly through inhibition of a downstream G protein. PACAP also inhibited potassium current in these cells, but in contrast to the PACAP-induced inward current, this effect was mimicked by cAMP analogues and was not modulated by Ras. These data suggest that coupling of PACAP to both intracellular second messenger systems, adenyl cyclase and phospholipase C, is required to activate the neurons of the adrenal medulla (3–6), where it regulates catecholamine synthesis and release (7–10).

Three receptor subtypes for PACAP have been identified, which display differential affinities for this peptide and specificity for VIP. The affinity of the type I receptor for PACAP is 3 orders of magnitude higher than for VIP. The type II receptor has a lower affinity for PACAP than the type I and is unable to discriminate between the two peptides. Whereas the type II receptor is exclusively coupled to adenyl cyclase, the type I receptor has dual coupling to both adenyl cyclase and phospholipase C (PLC) (11). Both of these receptors have been cloned and are predicted to conform to the classic seven-transmembrane domain pattern of G protein-coupled receptors. At least six splice variants of the type II receptor have been demonstrated, and these alter the precise pattern of coupling when expressed in heterologous systems (11–13). A third PACAP receptor, which is not coupled to adenyl cyclase or phospholipase C, appears to be expressed preferentially in pancreatic beta cells (14, 15).

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inward current associated with [Ca\(^{2+}\)]\(_i\), transients in PC12 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture reagents were purchased from Life Technologies, Inc. PACAP38 was from Peninsula Laboratories, Inc. (Belmont, CA) and was applied in extracellular solution containing a mixture of peptide inhibitors (10 μg/ml each of chymostatin, leupeptin, and antipain and 1 μM pepstatin A, all from Sigma). GTPγS, AMP-PCP, db-cAMP, and 8-Br-cAMP were from Sigma. (R)-cAMPs was from Biologic Life Science Institute (Bremen, Germany). Fura-2 AM, H89, pyrazone, and 6-

Cell Culture—PC12 and PC12asn17-W7 cells were plated on collagen-coated tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented as described previously (23) and grown at 37 °C in 5.0% CO\(_2\). The absence of active p21ras was inferred by a voltage step from around 5 megaohms, was compensated by series resistance, usually

Membrane currents were recorded under voltage clamp using an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz, digitized at 2.5–10 kHz using a DataQ 1200 interface (Axon Instruments), and stored on a computer using pCLAMP (version 5) software (Axon Instruments). Series resistance, usually around 5 megaohms, was compensated by ~80%. Input resistance and membrane capacitance were calculated from the current transient elicited by a voltage step from ~80 to ~90 mV. The PC12 cells studied had an input resistance of 17 ± 1.2 gigahm and capacitance of 2.7 ± 0.3 pF (n = 70). The PC12asn17-W7 cells had an input resistance of 1.2 ± 0.1 gigahm (n = 27) and capacitance of 10.8 ± 1 pF (n = 27). Currents were analyzed off-line using pCAMP (versions 5 and 6; Axon Instruments) and Origin (version 4; MicroCal, Inc., Northampton, MA) software.

Drug solutions were applied from a homemade, gravity-driven perfusion system, which exchanged the solution around a cell in 50 ms.

Effects of agents incorporated into the pipette solution were determined by recording alternately from cells with drug-free and drug-containing solutions. Results are expressed as mean ± S.E. Statistical analyses were performed using paired or unpaired Student’s t tests, with p ≤ 0.05 considered significant.

**RESULTS**

**PACAP Causes Ca\(^{2+}\) Influx in PC12 Cells by Activating a Ca\(^{2+}\)-Carrying Current**—As shown in Fig. 1A, PACAP induced a biphasic increase in [Ca\(^{2+}\)]\(_i\). The initial increase was followed by a fall in [Ca\(^{2+}\)]\(_i\), to a lower, sustained level, which was blocked by 1 mM CoCl\(_2\) (a). In the presence of 1 mM CoCl\(_2\), PACAP produced only a transient increase in [Ca\(^{2+}\)]\(_i\) (b). B, PACAP38 (1 μM) induced an inward current at ~ 80 mV, which was inhibited by 1 mM CoCl\(_2\) (a) and reduced in Ca\(^{2+}\)-free solution (b). C, relationship between membrane potential and the amplitude of the current induced by 1 μM PACAP38. Current amplitude is normalized against cell capacitance. Points and bars represent mean ± S.E. of 3–7 cells.

**FIG. 1. Influence of PACAP38 on intracellular [Ca\(^{2+}\)]\(_i\) and membrane current. A, PACAP38 (5 nM) produced a biphasic increase in [Ca\(^{2+}\)]\(_i\). The initial increase was followed by a fall in [Ca\(^{2+}\)]\(_i\), to a lower, sustained level, which was blocked by 1 mM CoCl\(_2\) (a). In the presence of 1 mM CoCl\(_2\), PACAP produced only a transient increase in [Ca\(^{2+}\)]\(_i\) (b). B, PACAP38 (1 μM) induced an inward current at ~ 80 mV, which was inhibited by 1 mM CoCl\(_2\) (a) and reduced in Ca\(^{2+}\)-free solution (b). C, relationship between membrane potential and the amplitude of the current induced by 1 μM PACAP38. Current amplitude is normalized against cell capacitance. Points and bars represent mean ± S.E. of 3–7 cells.**
rable among cells studied on a given day but varied widely from day to day, ranging from 10 pA to 10 nA at −80 mV, in response to 1 μM PACAP38. Moreover, a second application of PACAP38 often produced a smaller response than the first. These factors complicated the analysis of dose-response relationships. Comparisons of currents activated by different concentrations of PACAP38 were therefore made using the first responses recorded from matched cells on one day, and current amplitudes were normalized against cell capacitance to control for variation in cell size. In one set of cells, the current activated by 1 nM PACAP38 (6 ± 2 pA/pF, n = 7) was significantly smaller (p < 0.01) than the current activated by 100 nM (20 ± 4 pA/pF, n = 4). In contrast, in other cells, 100 nM PACAP38 activated a current (2 ± 1 pA/pF, n = 3) that was not significantly different from that activated by 1 μM PACAP38 (6 ± 3 pA/pF, n = 3). PACAP38 also induced comparable currents at 500 nM (8 ± 6 pA/pF, n = 3) and 5 μM (13 ± 9 pA/pF, n = 3), suggesting that the maximum response was reached by around 100 nM PACAP38.

As found with the sustained [Ca2+]i response, the current induced at −80 mV by 1 μM PACAP38 was reversibly inhibited by 1 mM CoCl2 (n = 4; Fig. 1B, a). The current induced by 1 μM PACAP38 was additionally reduced by exposure to Ca2+-free medium (Fig. 1B, b), although by only 25 ± 7% (n = 5). Removing Na+ from the medium, by equimolar substitution of tetraethylammonium chloride for NaCl, had a larger effect, reducing the current by 90% from 86 ± 43 pA (n = 9) to 8 ± 3 pA (n = 10; p < 0.05). In contrast, the amplitude of the PACAP-induced current was not significantly altered at any potential when the extracellular NaCl was replaced with equimolar Na2SO4 (n = 3) or the K+ in the pipette solution was replaced with equimolar Ca2+ (n = 4).

The voltage dependence of the current induced by 1 μM PACAP38 is shown in Fig. 1C. Pronounced inward rectification was apparent at negative potentials with negligible current recorded at positive potentials. When current could be measured at positive potentials, it was always inwardly directed (up to 40 mV), implying a positive reversal potential as expected for a current carried by Ca2+ or Na+.

**Intracellular Mediators of the PACAP38-induced Current**—To test the involvement of protein phosphorylation in current activation by PACAP38, phosphorylation was prevented by adding the nonhydrolyzable ATP analogue AMP-PCP (1 mM) to the pipette solution. In these conditions, 1 μM PACAP38 induced a significantly smaller current at −80 mV (Fig. 2A, a). The current induced at −80 mV by 1 μM PACAP38 was also significantly reduced when the PKA was blocked, either by adding (R906)-cAMPS (2 mM) to the pipette solution (Fig. 2A, a) or by adding H89 (50 μM) to the extracellular solution (Fig. 2A, b). The current induced by 1 μM PACAP38 was also suppressed by more than 50% in the presence of the PLC inhibitor, U73122 (50 μM), whereas the same concentration of U73343, an analogue of U73122 that lacks the inhibitory action on PLC, had no effect (Fig. 2A, b). The protein kinase C (PKC) inhibitor, RO318220, also reduced the current, but by only 19% at 100 μM (Fig. 2A, b).

Despite the inhibitory effects of PKA blockers on the PACAP38-induced current, membrane-permeable analogues of cAMP, which stimulate PKA activity, were unable to mimic the action of PACAP38 even at high concentrations. As illustrated in Fig. 2B for 10 mM db-cAMP, neither this analogue nor 8-Br-cAMP induced significant current at −80 mV, although in the same cells 1 μM PACAP38 did induce substantial current. These cAMP analogues did, however, mimic an inhibitory effect of PACAP38 on K+ currents recorded from PC12 cells (Fig. 2C).

**Outward K+ currents activated by steps from −80 to 40 mV were reduced by 50 ± 11% (n = 10) in the presence of 5 μM PACAP38. The same currents were reduced by 26 ± 3% (n = 5) in the presence of 10 mM db-cAMP and by 20 ± 10% (n = 5) in the presence of 10 mM 8-Br-cAMP.**

**Involvement of p21ras in the PACAP38-induced Current**—Studies on Drosophila muscle found that PACAP activation of inward current and modulation of K+ current required the co-activation of cAMP and Ras/Raf signaling pathways (26). We examined the involvement of p21ras in the response of PC12 cells to PACAP38, using a dominant negative Ras (PC12asn17-W7) cell line. This dominant negative form of Ras was previously shown to inhibit the classical Ras-dependent stimulation of extracellular signal-regulated kinase 1/2 by nerve growth factor (23). The application of PACAP38 to PC12asn17-W7 cells induced an inward current at negative membrane potentials (Fig. 3A), as observed in PC12 cells. The amplitude of the current induced by PACAP38 (5 μM, −80 mV) was, however, significantly (p < 0.03) larger in PC12asn17-W7 cells (Fig. 3B). In addition, the current activated with a delay of 26 ± 3 s (n = 5), which was significantly (p < 0.02) shorter than the delay of 49 ± 9 s (n = 7) measured in wild type PC12 cells (Fig. 3C). The inhibitory effect of PACAP38 on K+ current was also retained
The effects of PACAP38 in PC12asn17-W7 cells. A, PACAP38 (5 μM) induced inward current at ~80 mV, while db-cAMP (10 mM) had essentially no effect. B, the current induced by 5 μM PACAP38 was larger in PC12asn17-W7 cells than in wild type PC12 cells. C, the duration of the latent period between the start of PACAP38 (5 μM) application and the appearance of current was shorter in PC12asn17-W7 cells. D and E, the outward K⁺ current activated by voltage steps from ~80 to 40 mV was reduced by PACAP38 (5 μM) and db-cAMP (10 mM) in PC12asn17-W7 cells. The numbers of cells studied are indicated within bars. *, p < 0.05 compared with PC12 cells.

In PC12asn17-W7 cells (Fig. 3D), where 5 μM PACAP38 caused a reduction of 40 ± 5% (n = 7) at 40 mV.

The effects of cAMP analogues were unaffected by the presence of the dominant negative Ras. Although they failed to activate significant inward current at ~80 mV (Fig. 3A), 10 mM db-cAMP or 8-Br-cAMP reduced the outward current at 40 mV (Fig. 3E) by 18 ± 6% (n = 5) and 20 ± 10% (n = 3), respectively.

Modulation by GTPγS—G proteins are uncoupled from receptors by the binding of GTPγS, which is expected to cause a nonselective activation of G proteins in the cell. When 300 μM GTPγS was added to the pipette solution, it activated an inward current at negative membrane potentials (Fig. 4). This current was distinct from that induced by PACAP38, because it activates inward current at negative membrane potentials (Fig. 4). This current was distinct from that induced by PACAP38, because it activates inward current at negative membrane potentials (Fig. 4). This current was distinct from that induced by PACAP38, because it activates inward current at negative membrane potentials (Fig. 4). This current was distinct from that induced by PACAP38, because it activates inward current at negative membrane potentials (Fig. 4). This current was distinct from that induced by PACAP38, because it activates inward current at negative membrane potentials (Fig. 4).

The initial transient rise may have reflected Ca²⁺ influx in rat PC12 cells, because the PACAP38-induced increase in [Ca²⁺]i was little affected by CdCl₂ or nifedipine at concentrations causing complete block of L-type channels (30). Ca²⁺ influx was more likely mediated by the CoCl₂-sensitive inward current activated by PACAP38 in voltage-clamped PC12 cells. This current reversed direction in the voltage range expected for Ca²⁺ influx and was inhibited by removing either ion from the extracellular solution, implying that both ions carried the current. Since removal of intracellular K⁺ failed to alter the current, it may reflect cation
channels with selectivity for Na$^+$ and Ca$^{2+}$. PACAP38 was also found to activate a Na$^+$-dependent current in bovine chromaffin cells (21). Activation of cation channels by PACAP would directly mediate Ca$^{2+}$ influx and cause membrane depolarization, leading to the opening of voltage-gated Ca$^{2+}$ channels. This depolarizing action of PACAP would be potentiated by its inhibitory effect on K$^+$ current. Since L-type channels are one of several voltage-gated Ca$^{2+}$ channel subtypes expressed in PC12 and chromaffin cells (30, 31), variation in the sensitivity of the secretory PACAP response to L-type Ca$^{2+}$ channel antagonists may reflect differences in the relative expression of these channels.

Blockade by millimolar GTP$\gamma$S of PACAP38-induced current activation, and hence Ca$^{2+}$ influx, is consistent with the involvement of a G protein-coupled receptor. Current activation also involved protein phosphorylation, because it was prevented by the nonhydrolyzable ATP analogue AMP-PCP. The response required activation of the adenyl cyclase-PKA pathway, because two different PKA inhibitors prevented it. The failure of membrane-permeant analogues of cAMP to mimic this effect of PACAP38 in PC12 cells shows, however, that this pathway is not, in itself, sufficient to account for the response. This contrasts with the inhibitory effect of PACAP on K$^+$ current, which could be mimicked by cAMP analogues. As suggested for adrenal chromaffin cells (21), current activation by PACAP38 also appeared to require the PLC signaling pathway, because it was inhibited by the selective PLC blocker U73122 but not its inactive analogue U73343. Downstream activation of PKC is unlikely to play a major role, because although the PKC inhibitor RO318220 reduced the response, the effect was small and observed at high drug concentrations. The PLC pathway involved in the activation of Ca$^{2+}$ influx by PACAP appears, therefore, to be distinct from PKC.

Taken together, our data indicate that adenyl cyclase and PLC are both activated by PACAP to elicit an inward current in PC12 cells, so that inhibition of either pathway blocks the response. This finding is consistent with the known dual coupling of the type I PACAP receptor to the two pathways. The concentration dependence of the PACAP38-induced current is also consistent with the involvement of a type I receptor, as are previous studies showing the involvement of a type I receptor in the secretory response of PC12 cells to PACAP (24). Heterologous expression studies (11, 13, 32) have consistently shown that coupling of PACAP to phospholipase C through the type I receptor requires higher concentrations of agonist ($>$10 nM) than does activating adenyl cyclase ($<$1 nM). At the concentrations of PACAP38 required to activate the current in this study, both pathways would have been stimulated.

The findings reported here provide an explanation for previous conflicting observations on the role of adenyl cyclase in mediating [Ca$^{2+}$]-transients. Inhibitors of either PKA (19, 20, 33, 34) or PLC (12, 16, 17, 21, 35) pathways have been reported separately to block [Ca$^{2+}$]-transients in a variety of cell types. However, other studies that found that analogues of cAMP or forskolin could not mimic the effect of PACAP were interpreted as evidence against a role for the cAMP pathway in mediating the [Ca$^{2+}$]-response (16, 17, 27).

The small GTP-binding protein Ras has been implicated in the regulation of ion channel activities (36–39). Furthermore, co-activation of the Ras/Raf and cAMP signaling pathways was found to be necessary for the activation of inward current and modulation of K$^+$ current by PACAP38 in Drosophila muscle (26). This was not the case in PC12 cells, because both the activation of inward current and inhibition of K$^+$ current caused by PACAP38 were retained in cells expressing a dominant negative form of Ras. In fact, in these PC12asn17-W7 cells, PACAP38 activated a larger current with a shorter latency, suggesting that Ras may exert a tonic inhibitory influence on the current or on the signaling pathways that activate the current. Tonic regulation of Ca$^{2+}$ channels by Ras was previously found in sensory neurons, although in these cells it had a stimulatory effect (38). An inhibitory action of Ras was found in atrial cells, where it prevented the coupling of muscarinic receptors to K$^+$ channels (36). Since Ras can be activated by G$\gamma$ subunits of heterotrimeric G proteins and by Ca$^{2+}$ influx (40, 41), its inhibitory effect on the inward current would be reinforced in the presence of PACAP38, through activation of the G protein-coupled type I receptor. Alternatively, it is possible that Ras exerts its inhibitory effect only during stimulation of the PACAP receptor.

Interestingly, the intracellular application of 300 $\mu$M GTP$\gamma$S in wild-type PC12 cells mimicked the effect of the dominant negative Ras, in that it reduced the latency and potentiated the amplitude of the PACAP38-induced current. Since it failed to do this in PC12asn17-W7 cells where the response was already enhanced and accelerated, it is likely that this action of GTP$\gamma$S reflects activation of GTP-binding proteins downstream of Ras, which bypass its inhibitory effect. Interestingly, in PC12asn17-W7 cells, GTP$\gamma$S enabled the activation of inward current by db-cAMP, an effect not seen in wild-type PC12 cells. Since CoCl$_2$ blocked the db-cAMP-induced current, it was probably the same as the current activated by PACAP38. Since current activation by PACAP38 appeared to require the co-activation of the cAMP and PLC pathways, this suggests that the PLC pathway was active in the presence of GTP$\gamma$S. Ras may therefore have an additional inhibitory effect in PC12 cells that is not bypassed by activating downstream G proteins. Since this inhibitory effect is overcome in the presence of PACAP38, Ras itself must be under inhibitory control by a signaling pathway activated by the PACAP receptor.

Even in the presence of GTP$\gamma$S, PACAP38 was required to activate the Ca$^{2+}$-carrying inward current in both PC12 and PC12asn17-W7 cells. Although GTP$\gamma$S did activate an inward current at negative potentials in the absence of PACAP38, it was distinct from the PACAP-dependent current because it reversed direction near 0 mV and was not blocked by CoCl$_2$. This current resembled a GTP$\gamma$S-activated Cl$^-$ current previously identified in chromaffin cells (42), suggesting that the same current is present in chromaffin-derived PC12 cells. In summary, the data presented here indicate that Ca$^{2+}$ influx initiated by PACAP in PC12 cells requires the dual activation of both intracellular signaling pathways coupled to the type I receptor, namely adenyl cyclase and phospholipase C. The Ca$^{2+}$ influx pathway is additionally under the negative influence of Ras, although part of this inhibitory action can be overridden by GTP$\gamma$S, presumably acting on GTP-binding proteins that are downstream of Ras. Our data also suggest an additional inhibitory action of Ras that cannot be overcome by activating downstream G proteins. This effect must be exerted on the PLC pathway, on the adenyl cyclase pathway downstream of PKA, or on the channel itself.

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